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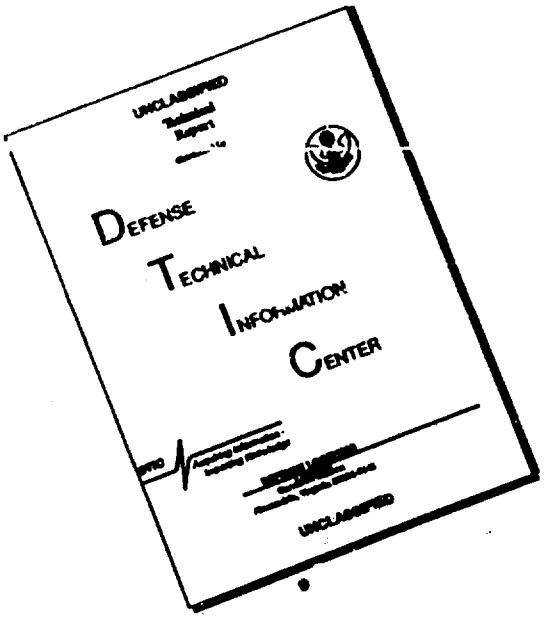

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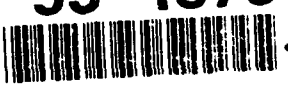

13. ABSTRACT *(Maximum 200 words)*

Amniote circadian organization derives from the interactions of circadian oscillators and photoreceptors located in the hypothalamic suprachiasmatic nuclei (SCN), the pineal gland, and the eyes. In mammals, circadian organization is dominated by the SCN, which serve as "master pacemakers" in the control of a wide array of behavioral and physiological rhythms (including locomotion, sleep-wake, thermoregulation, cardiovascular function, and many endocrine processes). Among the rhythms under SCN control in mammals are the circadian synthesis and secretion of the pineal hormone melatonin, which relies on a multisynaptic pathway via the sympathetic nervous system to maintain and entrain rhythmicity in this hormone. Several studies have indicated that pineal melatonin feeds back on SCN rhythmicity to modulate circadian patterns of activity and other processes. However, the nature and system-level significance of this feedback are unknown. Recently published work indicates that although pinealectomy does not affect rat circadian rhythms in light-dark cycles or constant darkness, wheel-running activity rhythms are severely disrupted in constant light. These data suggest that either (1) pineal feedback regulates the light sensitivity of the SCN, and/or (2) it affects coupling among

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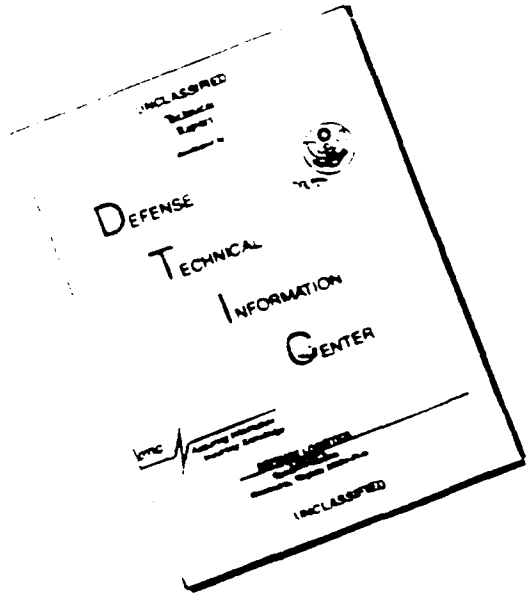

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Melatonin, the Pineal Gland, and Circadian Rhythms

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Abstract Amniote circadian organization derives from the interactions of circadian oscillators and photoreceptors located in the hypothalamic suprachiasmatic nuclei (SCN), the pineal gland, and the eyes. In mammals, circadian organization is dominated by the SCN, which serve as "master pacemakers" in the control of a wide array of behavioral and physiological rhythms (including locomotion, sleep–wake, thermoregulation, cardiovascular function, and many endocrine processes). Among the rhythms under SCN control in mammals are the circadian synthesis and secretion of the pineal hormone melatonin, which relies on a multisynaptic pathway via the sympathetic nervous system to maintain and entrain rhythmicity in this hormone. Several studies have indicated that pineal melatonin feeds back on SCN rhythmicity to modulate circadian patterns of activity and other processes. However, the nature and system-level significance of this feedback are unknown. Recently published work indicates that although pinealectomy does not affect rat circadian rhythms in light–dark cycles or constant darkness, wheel-running activity rhythms are severely disrupted in constant light. These data suggest that either (1) pineal feedback regulates the light sensitivity of the SCN, and/or (2) it affects coupling among circadian oscillators within the SCN or between the SCN and its output. Research in our laboratory is currently addressing each of these hypotheses.

Key words

Circadian rhythms in amniotes are generated by endogenous circadian oscillators in the hypothalamic suprachiasmatic nuclei (SCN), the pineal gland, and ocular retinae, and are entrained to daily light–dark cycles by specialized photoreceptors in the retinae, pineal gland and brain. The relative importance of each of these components varies among taxonomic groups (Takahashi and Zatz, 1982). In reptiles and birds, overt rhythmicity results from the integration of multiple circadian oscillators within the pineal gland, the eyes, and the presumed homologue of the mammalian SCN (Cassone and Menaker, 1984; Underwood and Goldman, 1987; Gwinner, 1989). These rhythms are entrained primarily by extraretinal photoreceptors in the pineal gland and brain. In mammals, the SCN serve as "master pacemakers" in the control of a wide array of behavioral and physiological rhythms, including locomotion, sleep–wake, thermoregulation, cardiovascular function, and many endocrine processes (Moore, 1983). Among the rhythms under SCN control in mammals are the circadian synthesis and secretion of the pineal hormone melatonin (*N*-acetyl,5-methoxytryptamine), which relies on a multisynaptic pathway via the sympathetic nervous system to maintain and entrain rhythmic synthesis and secretion of this hormone (Klein, 1979). Disruption of the pathway from the SCN to the pineal gland at any level—destruction of the SCN itself, knife cuts of SCN afferents, or pharmacological blockade of the sympathetic innerva-

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PINEALECTOMIZED RATS ENTRAIN AND PHASE-SHIFT TO MELATONIN INJECTIONS IN A DOSE-DEPENDENT MANNER

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Abstract

Previous work has shown that daily injections of the pineal hormone melatonin (N-acetyl, 5 methoxytryptamine) entrain the free-running locomotor rhythms of rats held in constant darkness (DD) (with a median effective dose [ED_{50}] of 5.45 $\pm$ 1.33 μg/kg) and in constant bright light (LL). The present experiments determined the dose-response characteristics of entrainment and phase-shifting to daily and single melatonin injections in both sham operated (SHAM) and pinealectomized (PINX) rats. The data indicated an ED_{50} of 332 $\pm$ 53 ng/kg and 121 $\pm$ 22 ng/kg for SHAM and PINX rats respectively during the entrainment experiment. The ED_{50}'s for the entrainment experiment were considerably lower than doses previously employed and much lower than doses employed in reproductive and metabolic studies in rats and hamsters. The data indicated that no partial entrainment occurred, nor were there differences in phase angle, length of activity or period among all effective doses. Next, a single injection of 1 mg/kg melatonin has previously been shown to cause a phase-advance of approximately 45 minutes when administered around circadian time (CT) 10. We found that both SHAM and PINX animals phase-advanced to a single melatonin injection given at CT10 in a dose-dependent manner. The data for the phase-shifting experiment indicated a median effective dose of 8.19 $\pm$ 0.572 μg/kg and 2.16 $\pm$ .326 μg/kg for SHAM and PINX animals respectively with an average phase advance of 40 minutes for both groups. Together, the data suggest that the presence of the pineal gland is not necessary for the effects of melatonin on the rat circadian system and that PINX animals are marginally more sensitive to melatonin than their SHAM operated controls.

INTRODUCTION:

Perhaps the best evidence that the pineal gland influences circadian clock function is the fact that daily administration of exogenous melatonin entrains circadian rhythms in several species of mammals (Armstrong et al. 1986; Darrow and Doyle 1990; Cassone et al 1986a; Illnerova 1991; Margraf et al. 1993; Redman et al. 1983; Thomas and Armstrong 1988), birds (Gwinner and Benzinger 1978; Cassone et al. 1992; Chabot and Menaker 1988) and reptiles (Underwood and Harless 1985). During entrainment of nocturnal rats to daily melatonin injections, the rest interval precedes the phase of injection or infusion while the activity interval follows it. In contrast to this, daily melatonin injections in diurnal reptiles and birds entrains circadian activity patterns such that the interval of activity precedes the time of injection while the interval of rest follows it.

In rats, melatonin has little effect on the circadian period or phase unless the time of injection exactly coincides with the animal's activity onset. The phase-relationship ($\Psi_{i,o}$) between the time of injection and the activity onset is invariant at 0 hours at all dosages (Cassone et al 1986a). During melatonin entrainment, activity onset continues to be phase-locked to melatonin injection until the injection regime is halted, at which time rats free-run with their own circadian period. This effect is dose-dependent with an ED_{50} of 5.3 $\pm$ 1.5 μg/kg (Cassone et al. 1986a). Further, phase-advances of about 30 minutes to an hour in response to single injections of 50 μg melatonin/kg body weight have been reported to occur around circadian time (CT) 10 (two hours before activity onset in DD) but not at other times (Armstrong and Chesworth 1987; Illnerova 1991). All of these data are consistent with the view that the circadian clock underlying rhythmicity, presumably the hypothalamic

suprachiasmatic nuclei (SCN), is entrained to melatonin injections by daily phase advances (Cassone 1990).

Underwood and Goldman (1987) have reasonably suggested that the unique phase relationship required for obtaining entrainment to melatonin injections in rats may involve a phase relationship between endogenous melatonin secreted by the pineal gland and the exogenously administered hormone. This phase relationship has also been suggested to be important in the reproductive effects of exogenous melatonin (Stetson and Watson-Whitmyre 1984; Stetson and Tate-Ostroff 1981; Tamarkin et al. 1976; Tamarkin et al. 1977; Watson-Whitmyre and Stetson 1983). To test this hypothesis, we have pinealectomized (PINX) rats in order to determine if entrainment to daily injections of melatonin occurs. We have also tested the effects of PINX on: 1) the dose-response characteristics of entrainment to daily injections of melatonin, and 2) the magnitude and dose-response characteristics of phase shifts produced by single injections of melatonin at CT 10.

MATERIALS AND METHODS:

Experiment 1. Adult male hooded Long-Evans rats (n=72) were maintained in a light-tight experimental room for 14 days during which food (Purina Rat Chow) and water were continuously available. They were housed in polycarbonate cages equipped with stainless steel tops. These cage tops were fitted with removable Plexiglas inserts, each of which contained a stainless steel running wheel (27 cm in diameter by 12 cm wide). Wheel-running revolutions were monitored by means of a data acquisition and control computer. Data were collected from magnetic microswitches attached to the running wheels with a Hewlett-Packard ES/12 Model 46 computer fitted with the Dataquest III (Mini-Mitter Co., Inc., Sunriver, OR) hardware and software package.

After 14 days, rats were anesthetized and either were surgically pinealectomized (n=36; PINX) or received a sham pinealectomy (n=36; SHAM). Rats were preanesthetized with methoxyflurane fumes and anesthetized with intramuscular injections of 80 mg/kg ketamine and 20 mg/kg xylazine in 0.9% saline (1 μl/g). They were then fitted into a stereotaxic instrument, and a cranial incision was made to expose the pineal area. In PINX rats, the dura mater was cut and pulled back to expose the pineal gland, which was then removed with fine mouse-toothed forceps. The dura and the skull cap were replaced and the incision was closed. SHAM rats experienced identical surgical procedures, with the exception that the pineal gland was not actually removed. After surgery, the incisions were treated with a topical antibacterial powder (nitrofurazone).

Rats were allowed to recover in the same LD 12:12 for an additional 14 days before being phase-delayed 8 hours. Cages were changed every 21 days during the experiment.

After 14 days in this new photoperiod, rats were placed in constant darkness (DD). Melatonin solutions were prepared using 1% ethanol in 0.9% saline. Paired groups of SHAM and PINX rats (n=4/group) received daily injections 0.4-0.6 ml of one of 9 dosages of melatonin at the same time daily, with the first injection given 4 hours after activity onset. An infrared viewer was used to administer the injections. The doses of melatonin employed were 1 mg/kg, 100 μg/kg, 50 μg/kg, 10 μg/kg, 1 μg/kg, 0.5 μg/kg, 0.1 μg/kg, 0.01 μg/kg, and 0.001 μg/kg. The injections were given subcutaneously. After 42 days of injections, rats were allowed to free-run 15 days. The guide/rule command in the TAU program (Mini-Mitter Co., Inc., Sunriver, OR) was used to analyze each ten day period during the injection regime. Animals were defined as entrained by two criteria: 1) the periods of their activity onsets (ϕ_o) became exactly twenty-four hours during the melatonin injection regime, and 2) they must express a free-running circadian rhythm after injections were ceased with a ϕ_o related to the time of injection (ϕ_i). Twelve (3 SHAM and 9 PINX) of the 72 rats did not free-run with a period that allowed activity onset to reach the time of injection, and were not included in the dose-response analysis unless the period of ϕ_o became exactly 24 hours during injections. τ_{bef} and τ_{aft} (see Tables 1 and 2) were determined using the guide/rule command of the TAU program. τ_{bef} was determined using the first 10 days into DD, and τ_{aft} was determined using the 10 days immediately following the last day of injections. Phase angles ($\Psi_{i,o}$) of activity onsets relative to time of injection were measured for each ten day period during the injection regime for each animal. The lengths of activity phases (α) were measured similarly. Probit analysis (Goldstein 1964) was used to determine the median effective doses ($ED_{50's}$) and their 95% confidence intervals. Statistically significant

differences between median effective doses were determined using Student's t test (Goldstein 1964).

Following these procedures, all rats were anesthetized with a cocktail of 80 mg/kg ketamine; 20 mg/kg xylazine and perfused transcardially with cold 0.9% saline and then with 0.1 M phosphate buffer (pH 7) containing 10% sucrose. Rats were then decapitated and carefully craniotomized to visually determine the presence or absence of pineal tissue. Brains were then removed and frozen in cold (-40° C) isopentane. They were then coronally sectioned on a cryostat at 20 μm through the pineal area. Sections were thaw-mounted to gelatin-coated slides and stained with cresyl violet for further histological examination to ascertain the completeness of the surgeries.

Experiment 2. Adult male-hooded Long-Evans rats (n=48) were maintained then pinealectomized (n=24) or received a SHAM surgery (n=24) as above. These animals were allowed to free-run for 14 days in DD before receiving one of eight dosages of melatonin at the animals' circadian time 10 (two hours prior to activity onset - circadian time 12). One group received saline in 1% ethanol injections. Circadian time 10 (CT10) was determined using activity plots over 14 days prior to the single injection and predicting CT12 on the day of injection. The doses employed were 1000, 100, 50, 10, 1, 0.5, 0.1, and 0.01 μg/kg melatonin; and a saline injected group (n=4-6/group).

Data from ten days before the injection were plotted on a signal averaging histogram by folding activity measures over the free running period (τ). The calculated τ was used as the x-axis so that an animal with a free running period greater or less than 24 hours would have activity measures exactly overlapped. τ was determined by plotting the time of each

day's onset as determined from the actogram, and, using a linear regression through these points, an equation was obtained that was used to predict τ. τ was confirmed using least squares cosinor analysis (Koopman 1974). The ten days following the injection were also plotted on the same histogram, and the difference in onsets was determined using both the actograms and histograms. The day of injection was omitted because the rats were active immediately following the injection, and this would have caused the resulting phase-shifts ($\Delta\phi$) to appear artifactually larger than the true steady state $\Delta\phi$. Although offsets appear to phase-advance along with onsets, they were not as precise as ϕ_o. Therefore, only $\Delta\phi_o$'s were used to analyze the phase-shifts.

Probit analysis was performed on the number animals that shifted to each dose to obtain ED_{50}'s and 95% confidence intervals. An average amount of shift for each group (SHAM and PINX) was determined using only those animals that responded to the single injection of melatonin. Statistical significance between groups was determined using Student's t-test (Goldstein 1964).

RESULTS:

Experiment 1: The results of the dose-response experiment to daily injections are summarized in Tables 1 and 2. Doses of melatonin from 1000 μg/kg to 1 μg/kg were 100% effective in entraining SHAM free-running rats in DD (Fig 1A). Doses below 0.5 μg/kg were ineffective (Fig 2A), and three of four animals entrained to 0.5 μg/kg. Doses from 1000 μg/kg to 10 μg/kg were 100% effective in entraining PINX operated animals (Fig 1C). Doses below 0.01 μg/kg were ineffective (Fig 2C), and a varying proportion of the PINX operated rats entrained to dosages between 1 μg/kg and 0.01 μg/kg. Probit analysis of the responses indicated that the ED_{50} for entrainment to subcutaneous melatonin injections for SHAM operated animals was 332 $\pm$ 53 ng/kg and for PINX rats 121 $\pm$ 22 ng/kg ($P<.05$).

Unlike previous reports (Cassone et al. 1986a,b), a positive $\Psi_{i,o}$ to the melatonin injections was seen in most of the entrained animals (Fig 1B,D) (Table 1 and 2). No differences were observed between the SHAM and the PINX group as $\Psi_{i,o}$ was 0.962 $\pm$ 1.4 hr. and 0.702 $\pm$ 1.1 hr. respectively. These positive phase-angles along with longer periods of activity (α) were not observed in other entrainment studies (Redman et al. 1983; Cassone et al. 1986a,b; Thomas and Armstrong 1988). The average α for the SHAM and PINX groups was 13.98 $\pm$ 0.44 hrs and 13.79 $\pm$ 0.47 hrs respectively as compared to values of approximately 12 hrs in the previous studies. Another interesting observation, not common to Long-Evans rats, is the presence of free-running periods shorter than 24 hours in several animals (Fig 2B,D). These animals could not be included in the dose-response characterization because their activity onset never coincided with the time of the melatonin injection. Figure 2D shows an animal with a short free-running period and an interesting

masking effect of the daily melatonin injections.

Experiment 2: The results of the phase-shifting single injections of melatonin are reported in Table 3 and in Figure 3. Rats phase-advanced 30-50 minutes to a single injection or they did not phase shift at all (Data collection restricted temporal resolution to 10 minute intervals). Varying proportions of rats phase-shifted to dosages between 100 μg/kg and 0.5 μg/kg in both SHAM and PINX rats (Fig 3A,B). Dosages below 0.5 μg/kg were ineffective in both groups as was saline (Fig 3C,D). Probit analysis of the number of animals that phase-shifted to the single injections indicated that the ED_{50} for the SHAM group was 8.19 $\pm$ 0.57 μg/kg and 2.16 $\pm$ 0.33 μg/kg for the PINX group ($P<.05$). Finally, the average amount of shift of animals that responded was 38.68 $\pm$ 15.35 (N=19) and 38.33 $\pm$ 7.28 (N=18) for SHAM and PINX rats respectively (not significant).

DISCUSSION:

We report here that the secretion of melatonin by the pineal gland is not necessary for rats to entrain to daily melatonin injections as PINX rats entrain similarly to their SHAM controls. The present results indicate that entrainment of free-running rats to daily injections of melatonin is dose-dependent in both SHAM and PINX rats, and that the ED_{50} for this effect is higher in SHAM rats (332 $\pm$ 53 ng/kg) than in PINX rats (121 $\pm$ 22 ng/kg). No significant differences in the length of α, $\Psi_{i,\infty}$, τ_{bef} or τ_{aft} were found among effective doses in either group. These observations along with the observation that both PINX and SHAM rats phase advance to a single injection of melatonin at CT10 similarly indicate that endogenous melatonin is not necessary for the behavioral effects of exogenous melatonin.

The ED_{50} values published in previous entrainment experiments are much higher than the values that we report here (5 μg/kg versus 100-300 ng/kg). Although we do not report here blood levels of the hormone following injection, a previous study (Cassone et al. 1986a) indicated that injection of 1 μg/kg melatonin (approximately ten times the ED_{50} here) resulted in peak serum melatonin titers of approximately 600 pg/ml within 10 minutes following injection. These values are approximately 10 times peak nocturnal levels in this species (Brown et al. 1982). Therefore, although the route of administration is by definition "pharmacological", the dosages employed are probably close to the "physiological range". This difference in ED_{50} could be due to strain differences or to differences in the experimental protocol. Rats employed in the present study, for example, expressed shorter τ's (SHAM: 24.15 $\pm$ 0.04 hrs and PINX: 24.13 $\pm$ 0.05 hrs) than did rats in previous studies

(24.23 ± 0.03 hrs - Cassone et al. 1986a). The shorter free-running periods indicate that melatonin injections phase-shifted the activity rhythm less than in the previous study in order to entrain the pattern. Secondly, in other entrainment studies, rats were allowed to free-run in DD for up to 25 days before the injections began. In the present study, injections were begun one day into DD, four hours after lights off. This difference in protocol may also have altered the ED_{50}. The ED_{50}'s for the phase shift experiment are much higher (2-8 μg/kg versus 100-700 ng/kg) than those of the entrainment study. This may be due to data analysis problems (it was difficult to determine phase-shifts of less than 20 minutes). It may also be that since these animals were in DD much longer (14 days) than in the entrainment study before the single injections were given, they were less sensitive to the hormone. In fact, the ED_{50}'s for the phase shift experiment correspond more closely with the previous dose-response entrainment study of Cassone and co-workers (1986a), where animals were allowed to free-run for 25 days before the injection regime.

The ED_{50} values using probit analysis for SHAM and PINX entrainment and phase-shifts suggest that the absence of the pineal gland may contribute to a slightly increased sensitivity to exogenous melatonin. PINX hamsters have been shown to be more sensitive than their SHAM operated controls in reproductive responses to exogenous melatonin (Goldman et al. 1979). Some supersensitivity should not be surprising since recent data have shown that melatonin receptors are probably coupled to a pertussis toxin sensitive G-protein (Carlson et al. 1989; Morgan et al. 1989; Laitinen and Saavedra 1990). The phenomenon of up and down regulation of receptors coupled to a G-protein is well known (Sibley et al. 1985; Weiss et al. 1988; Gilman 1987). Further, the density of melatonin

receptors increases in rodent pars tuberalis and SCN in response to PINX and constant light (Gauer et al. 1992; Gauer et al. 1993). It is possible that an up-regulation (increase in Bmax) may cause an increase in the sensitivity to exogenous melatonin in PINX rats, although these data are not currently available.

Unlike other entrainment studies, we report positive phase-angles to the daily injection regime so that many of these rats (both PINX and SHAM) were in their wheels an hour before the injections were given. While some animals anticipated the daily injection with a positive phase-angle of greater than three hours, the average $\Psi_{i,o}$ is approximately one hour for both PINX and SHAM rats (Tables 1 and 2). This could be due to the very short free-running periods of these animals or to their relatively higher sensitivity to the hormone (cf. Pittendrigh 1981).

These positive phase-angles also suggest that these rats are entraining and phase-shifting directly to melatonin and not to the activity induced by handling. Presently over twenty substances that can alter either the period or phase of free-running circadian rhythms in mammals have been found (For reviews, see Takahashi and Zatz 1982; Turek 1987,88; Turek and Van Reeth 1988; Wirz-Justice 1987). In addition to these pharmacological manipulations, elevation of activity itself may alter clock function. Many authors have also found changes in τ (Aschoff et al. 1973; Pratt and Goldman 1986; Yamada et al. 1988) and ϕ (Mrosovsky and Salmon 1987) associated with changes in levels of activity or excitement.

Perhaps the most intensely studied of these drugs is triazolam. Single (Turek 1989) and daily (Turek and Van Reeth 1988; Turek and Losee-Olsen 1987) injections of triazolam cause phase-shifts dependent upon the circadian time of drug administration. With only a

few exceptions in diurnal squirrel monkeys (Mistlberger et al. 1988) and humans (Van Cauter et al. 1987) triazolam's effects on the circadian clock are related to the increased locomotor activity induced by the drug. Physical restriction of triazolam-treated hamsters blocks the effect of the drug (Turek 1989). Recently, Hastings et al. (1992) showed that Syrian hamsters phase-advance to manual injections of melatonin and saline equivalently and have suggested that the effect of melatonin may also be activity dependent. We do not believe this to be the case for several reasons. First, as stated above, many of these animals are active in anticipation of the injection each day. Therefore, the daily phase-advance cannot be caused by changes in level of activity. One would also predict entrainment for all rats if arousal alone is causing entrainment. However, in this and previous studies (Cassone et al. 1986a, Thomas and Armstrong 1988) daily injections of saline and sub-threshold dosages of melatonin are ineffective. In the single injection experiment, the level of activity was elevated near ϕ_o after the single injection. However, doses below 0.5 μg/kg and saline did not have any effect on ϕ or τ suggesting that it is melatonin and not feedback from increased activity that caused the phase-shift. Therefore, we believe that the behavioral effects of melatonin in the present study result from melatonin acting directly at the clock - not through a feedback mechanism involving activity.

Although this study provides no direct evidence that melatonin's site of action is the SCN, we believe that entrainment and phase-shifting are occurring due to a direct effect of melatonin within the SCN. Many authors have shown that the SCN are physiologically sensitive to exogenous melatonin *in vivo* (Cassone et al. 1987; Cassone et al. 1988) and *in vitro* (Yu and Rusak 1993; Margraf et al. 1993; Mason and Rusak 1990; McArthur et al.

1991; Shibata et al. 1989; Vanecek et al. 1987; Weaver et al. 1989). Furthermore, the SCN of several species of mammals contain high affinity melatonin binding sites (Stankov and Reiter 1990; Stankov et al. 1993). Finally, electrolytic ablation of the SCN prevents entrainment to injections of melatonin (Cassone et al. 1986b). Alternatively, but not exclusively, the retinae may also contribute as sites of melatonin action here. The neural retinae also contain melatonin binding sites (Dubocovich et al. 1986) and melatonin affects mammalian (Dubocovich 1985) retinal physiology. However, enucleated rats have been reported to entrain to melatonin regimes (Chesworth cited in Armstrong 1989), as have blind human patients (Sack et al. 1991). Furthermore, enucleation has no effect on the inhibitory action of melatonin on SCN 2-deoxy-[1-^{14}C]glucose uptake (Cassone et al. 1987), and the several reports of melatonin sensitivity in vitro strongly suggests the eyes are not necessary for this effect.

In addition to this evidence of melatonin's action, several authors have demonstated that the pineal gland plays some role in mammalian circadian organization, although that role is at present unclear. Although PINX has very little effect on the free-running circadian locomotor rhythms of mammals in constant darkness (DD) (Richter 1967; Quay 1968; Aschoff et al. 1982) and dim constant light (Cheung and McCormack 1982), PINX of rats (Quay 1970; Armstrong and Redman 1985) and hamsters (Finkelstein et al. 1978) affects the rates of re-entrainment during large shifts of photoperiod. Some authors (Aschoff et al. 1982; Rusak 1982) have suggested that PINX has no direct effect on the circadian system but instead only increases the impact of sensory input, causing "masking" of the behavioral output of the circadian clock. Recently, however, research in our laboratory has shown that

PINX disrupts the circadian patterns of locomotion of rats held in constant light (LL) but not in DD (Cassone 1992). Further, Aguilar-Roblero and Vega-González (in press) indicate a clear effect of PINX on the time course of the splitting of wheel running circadian rhythmicity in hamsters. Rusak and Yu (1993) report that PINX one week prior to recording from suprachiasmatic nucleus cells in hamster brain slices eliminated the daily rhythm of melatonin responsiveness and altered the firing-rate rhythm. The morning peak was not sustained, and the nocturnal trough was attenuated. Taken together, the data support the idea that the mammalian SCN is sensitive to melatonin, and that this sensitivity is somehow important to overt circadian organization.

In conclusion, the hypothesis of Underwood and Goldman (1987) that the distinct phase of melatonin sensitivity derives from the coincidence of exogenous melatonin administration and the endogenous hormone being secreted during subjective night is disproved in the present study. We demonstrate that the presence of the pineal gland is not necessary for the behavioral effects of exogenously administered melatonin. PINX rats are able to entrain to daily injections of melatonin and do so in a manner identical to that of their SHAM operated controls. Further, PINX rats also phase-shift to a single injection of melatonin given at CT 10 in exactly the same way as SHAM animals. Finally, we find that a marginal supersensitivity to melatonin is produced by pinealectomy.

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Table 1 - EFFECTS OF DAILY MELATONIN INJECTIONS ON SHAM RATS

Dose (μg/kg)	τ_{bef} (hr $\pm$ SD)	τ_{aft} (hr $\pm$ SD)	α_{cont} (hr $\pm$ SD)	$\Psi_{i,o}$ (hr $\pm$ SD)	Entrain/N	Probit
1000	24.15 $\pm$ 0.03	23.98 $\pm$ 0.09	13.91 $\pm$ 0.35	0.98 $\pm$ 0.67	4/4	--
100	24.06 $\pm$ 0.06	24.09 $\pm$ 0.04	14.54 $\pm$ 0.61	0.96 $\pm$ 1.91	4/4	--
50	24.17 $\pm$ 0.10	24.03 $\pm$ 0.01	14.43 $\pm$ 0.74	1.26 $\pm$ 2.52	4/4	--
10	24.20 $\pm$ 0.04	24.04 $\pm$ 0.06	14.21 $\pm$ 0.46	0.00 $\pm$ 0.00	2/2	--
1	24.14 $\pm$ 0.08	24.04 $\pm$ 0.04	13.42 $\pm$ 0.52	1.20 $\pm$ 1.83	4/4	7.31
0.5	24.16 $\pm$ 0.05	24.07 $\pm$ 0.06	13.97 $\pm$ 0.23	0.69 $\pm$ 0.17	3/4	5.63
0.1	24.20 $\pm$ 0.04	24.13 $\pm$ 0.07	14.21 $\pm$ 0.09	--	0/4	2.68
0.01	24.15 $\pm$ 0.04	24.13 $\pm$ 0.06	13.18 $\pm$ 1.13	--	0/4	--
0.001	24.16 $\pm$ 0.06	24.06 $\pm$ 0.14	13.96 $\pm$ 0.38	--	0/3	--

Table 1 - Values for τ, α and Ψ are given as well as numbers of entrained animals and calculated probits for the SHAM operated animals according to dosage.

Table 2 - EFFECTS OF DAILY MELATONIN INJECTIONS ON PINX RATS

Dose (μg/kg)	τ_{bef} (hr $\pm$ SD)	τ_{aft} (hr $\pm$ SD)	α_{ent} (hr $\pm$ SD)	$\Psi_{i,o}$ (hr $\pm$ SD)	Entrain/N	Probit
1000	24.18 $\pm$ 0.06	24.03 $\pm$ 0.08	14.16 $\pm$ 0.46	0.00 $\pm$ 0.00	4/4	--
100	24.12 $\pm$ 0.03	24.01 $\pm$ 0.09	13.94 $\pm$ 0.08	0.93 $\pm$ 0.70	2/2	--
50	24.10 $\pm$ 0.01	24.03 $\pm$ 0.01	13.86 $\pm$ 0.66	1.31 $\pm$ 0.53	4/4	--
10	24.09 $\pm$ 0.00	24.07 $\pm$ 0.00	13.67 $\pm$ 0.00	0.00 $\pm$ 0.00	1/1	7.06
1	24.15 $\pm$ 0.05	24.04 $\pm$ 0.11	13.38 $\pm$ 1.41	0.22 $\pm$ 0.31	2/3	5.39
0.5	24.17 $\pm$ 0.07	24.08 $\pm$ 0.08	13.86 $\pm$ 0.84	0.00 $\pm$ 0.00	2/4	4.96
0.1	24.11 $\pm$ 0.01	24.01 $\pm$ 0.11	14.07 $\pm$ 0.74	0.47 $\pm$ 0.00	1/3	4.57
0.01	24.05 $\pm$ 0.05	23.99 $\pm$ 0.09	12.79 $\pm$ 0.62	3.39 $\pm$ 0.00	1/2	5.41
0.001	24.10 $\pm$ 0.07	23.98 $\pm$ 0.15	14.43 $\pm$ 0.63	--	0/4	2.77

Table 2 - Values for τ, α and Ψ are given as well as numbers of entrained animals and calculated probits for the PINX operated animals according to dosage.

Table 3 - PHASE-SHIFTING EFFECTS OF MELATONIN

Dose (μg/kg)	#Shifted/N (SHAM)	Average Shift (min) (SHAM)	Working Probit (SHAM)	#Shifted/N (PINX)	Average Shift (min) (PINX)	Working Probit (PINX)
1000	6/6	41.7	--	5/6	31.7	5.956
100	5/6	35	5.926	4/5	32	5.820
50	3/5	24	5.244	2/4	18.7	4.960
10	2/4	15	5.000	3/4	26	5.645
1	2/6	6.7	4.570	3/6	20	5.000
0.5	1/5	9	4.160	1/3	13	4.565
0.1	0/4	0	--	0/3	0	--
0.01	1/4	10	--	0/4	0	--
Saline	0/6	0	--	0/4	0	--

Table 3 - Values for the number of animals that responded, the amount of the phase shift and calculated probits are given for SHAM and PINX operated animals.

Figure Legends

Figure 1: SHAM AND PINX RATS ENTRAIN TO DAILY MELATONIN INJECTIONS

Double plotted actograms of rats held in LD 12:12 (block 1) followed by an eight hour phase delay (block 2). Rats were then placed in DD. The straight line through the actograms is drawn to show the time of injections each day for 42 days (asterisk indicates the day of last injection). [A] and [C] show a SHAM and PINX rat respectively that entrained to daily injections of melatonin (1 μg/kg) with a $\Psi_{i,o}$ of 0 hrs. [B] and [D] are SHAM and PINX animals respectively that entrained to daily injections of 10 μg/kg melatonin with a positive $\Psi_{i,o}$.

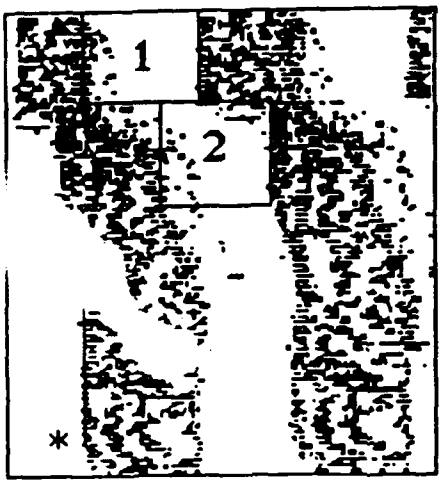
Figure 2: PINX AND SHAM RATS THAT DO NOT ENTRAIN TO DAILY MELATONIN INJECTIONS

Double plotted actograms of rats held in LD 12:12 (block 1) followed by an eight hour phase delay (block 2). Rats were then placed in DD. The straight line through the actograms is drawn to show the time of injections each day for 42 days (asterisk indicates the day of last injection). [A] and [C] show a SHAM and PINX rat respectively that did not entrain to daily injections of melatonin (0.001 μg/kg) with a τ of greater than 24 hrs. [B] and [D] show a SHAM and PINX rat respectively that were not used in the dose-response analysis because their activity onsets never reached the time of injection. An interesting masking effect of injections is seen in [D].

Figure 3: PINX AND SHAM RATS PHASE-ADVANCE TO SINGLE INJECTIONS OF MELATONIN

The single plotted actograms here show wheel-running activity several days of DD before and after a single injection of melatonin at CT 10 (asterisk). Notice the phase-advance in [A] and [B] and the lack of phase-shift in [C] and [D]. [A] and [B] are SHAM and PINX animals respectively that received a single injection of 10 μg/kg melatonin. [C] and [D] are SHAM and PINX animals that received a single injection of saline.

A



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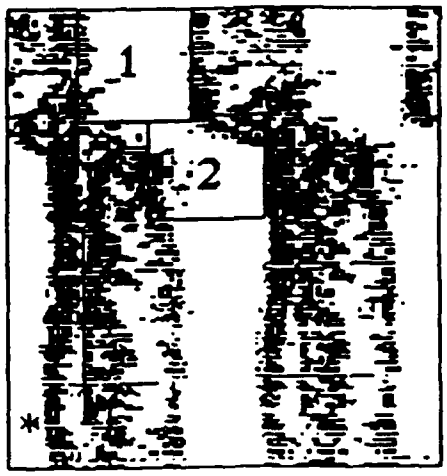

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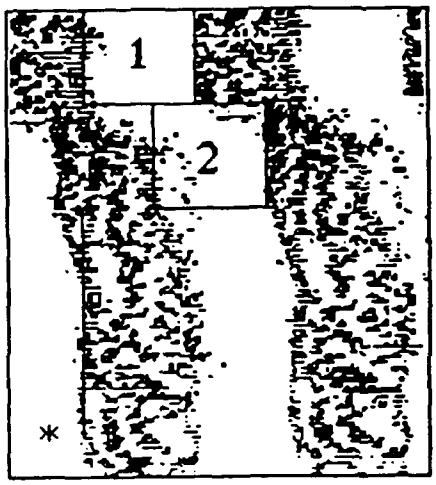

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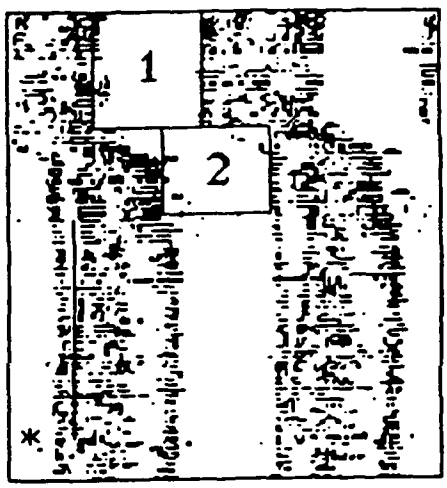

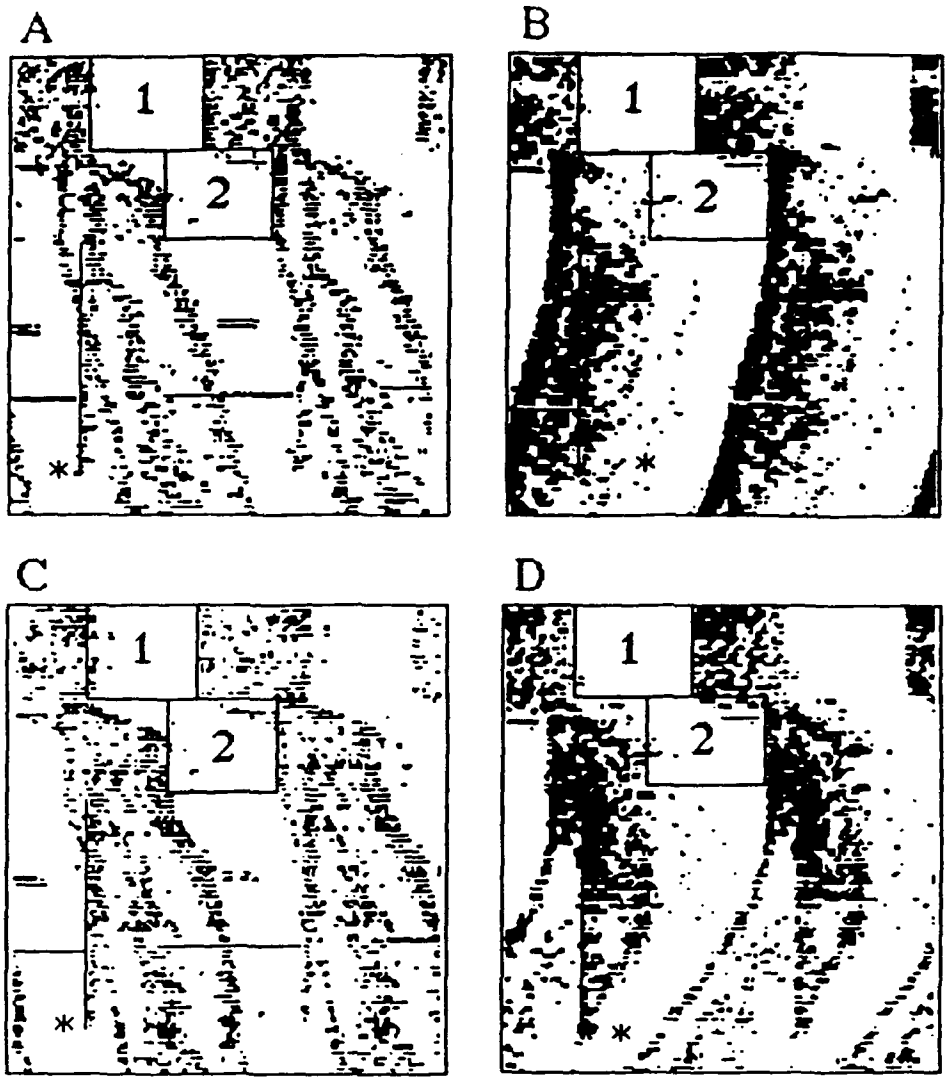
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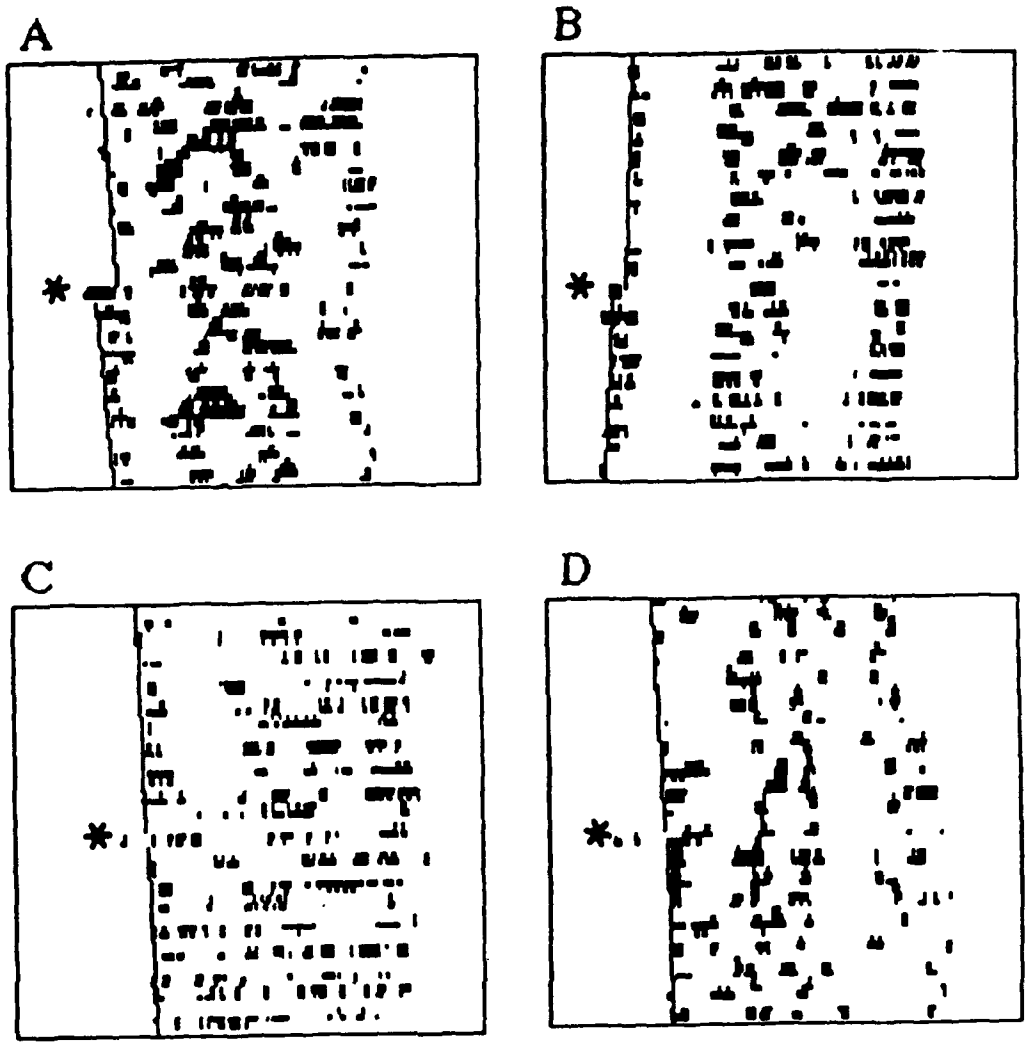
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THE SUPRACHIASMATIC NUCLEUS CONTROLS THE CIRCADIAN RHYTHM OF HEART RATE, BUT NOT OF ACTIVITY AND BODY TEMPERATURE, VIA THE SYMPATHETIC NERVOUS SYSTEM

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SUBMITTED TO AM. J. PHYSIOL.

Abstract

The mammalian suprachiasmatic nucleus (SCN) is the master pacemaker controlling a wide variety of circadian behavioral and physiological processes. However, the specific motor output pathway(s) by which these diverse processes are controlled are unknown. The only established motor output of this system is the regulation of pineal melatonin synthesis via the sympathetic nervous system. It is therefore possible that other peripheral circadian rhythms are regulated by this same system. To address this issue, surgically implanted transmitters with computer data acquisition were used to simultaneously record body temperature (BT), general activity (GA), wheel running activity (WR) and heart rate (HR) in the laboratory rats. Two experiments were performed: 1) the effects of SCN lesion (SCNX; N=5) vs sham-operated (SHAM; N=5) and 2) the effects of sympathectomy with the drug guanethidine (GUAN; N=7) vs saline control (SAL; N=5). SCNX abolished circadian patterns in all motor outputs, while SHAM animals showed robust rhythms in all measures. These data indicate that all circadian rhythms measured were generated by the SCN, including BT, and that residual rhythmicity was the result of incomplete lesions. In contrast, guanethidine, which depleted peripheral but not central catecholamine content, selectively abolished HR rhythmicity. This drug decreased HR circadian power to a level similar to the decrease caused by SCNX. Other rhythms (BT, GA and WR) were unaffected. These results suggest that the SCN influences some peripheral targets via circadian regulation of the sympathetic nervous system, while other circadian outputs are regulated via different, unknown pathways.

Introduction:

In mammals, the suprachiasmatic nucleus (SCN) serves as the master pacemaker controlling a wide array of behavioral and physiological rhythms including locomotion, sleep/wake, thermoregulation, cardiovascular function and many endocrine processes (35, 47, 69). Despite the fact that many rhythmic outputs have been shown to be under SCN control, the nature of the circadian signals generated and the mechanism(s) of transduction of this rhythmic information by the SCN to these processes are unknown.

The anatomical connection between the SCN and its rhythmic control of pineal melatonin release remains the only well characterized output pathway. SCN neurons have dense projections to the region just ventral of the paraventricular nucleus of the hypothalamus (PVN) with some fibers extending into the PVN itself (5, 58, 64, 65). Cells within and/or just ventral to the PVN project to regions of the intermediolateral cell column of the thoracic spinal cord that innervate the superior cervical ganglion (SCG) (6, 16, 44, 45, 59). Post-ganglionic fibers from the SCG regulate the rhythmic production of pineal melatonin through sympathetic innervation (27).

Several lines of evidence suggest that the SCN may have a role in regulating many homeostatic mechanisms through control of the autonomic nervous system (2, 24, 27, 42, 48). Among the many autonomically innervated peripheral tissues that exhibit circadian rhythmicity (4, 31, 39, 49), one of the most prominent is the circadian rhythm of heart rate. Moreover, several factors related to cardiovascular disease have been shown to be under sympathetic control in humans (37, 40, 36).

Although many aspects of autonomic outflow pathways are precise and differentiated

(25), the overall effect of the sympathetic system is to decrease activity in the visceral organs and to stimulate the heart and somatic muscles in a concerted fashion preparing the animal for "fight-or-flight" (11). In contrast, the ganglion cells of the parasympathetic system have local effects related to facilitating the activities of their respectively innervated organs (11, 23). The fact that the sympathetic nervous system acts in concert suggests that the SCN may be relaying rhythmic information to numerous downstream homeostatic processes via a multisynaptic pathway including the sympathetic chain.

The present experiments measured four rhythmic outputs (wheel running, general activity, body temperature and heart rate) in order to confirm SCN control of circadian rhythmicity. After establishing that the SCN controls heart rate rhythmicity, we ask if pharmacological blockade of sympathetic activity can disrupt the circadian pattern of any or all of these rhythms using the drug guanethidine to block the release of neurotransmitter from peripheral noradrenergic neurons.

Materials and Methods:

Effects of SCN Ablation on Multiple Circadian Outputs

Adult male hooded Long-Evans rats (n=12) were maintained in polycarbonate cages equipped with stainless steel tops in a light-tight experimental room where food (Purina Rat Chow) and water were continuously available. They were anesthetized with intramuscular injections of a cocktail consisting of 80 mg/kg ketamine and 20 mg/kg xylazine in saline (1 μl/g). Model #TA10CTA-F40 transmitters (Mini-Mitter Co.,Sunriver, OR) were implanted subcutaneously into each rat in order to simultaneously measure general activity, body temperature and heart rate. The receivers (Model #RA1010; Mini-Mitter Co., Inc., Sunriver, OR), placed under each cage, provided a digital representation of the telemetered signal in a format compatible with Hewlett-Packard ES/12 Model 46 computer fitted with Dataquest III (Mini-Mitter Co., Inc., Sunriver, OR) hardware and software package.

Animals were allowed to entrain for two weeks to a 12:12 light:dark (LD) cycle before 7 received electrolytic lesions to the SCN (4 milliamps for 10 seconds; SCNX) and 5 received a SHAM surgery (the electrode was placed into the SCN and no current was applied). Stereotaxic lesions were aimed at the SCN according to the atlas of Pellegrino, Pellegrino and Cushman (1979). The rats were allowed to recover in the previous light:dark cycle for 7 days before being placed into constant dim light (LL) (10^{14} quanta/sec/cm^2) for 14 days.

At the end of the experiment animals were anesthetized as described above and transcardially perfused with physiological saline. Fixation was accomplished with 4% paraformaldehyde in 0.1 M phosphate buffer (Ph = 7.4) with 10 mM sodium periodate and

75 mM lysine (33). Brains were removed and postfixed for 2 hr by placing them into the above solution. After postfixation, brains were cryoprotected with a successively increasing sucrose gradient, 10% (1 hr), 20% (1 hr), and 30% sucrose in 0.1 M phosphate. Serial 30-μm coronal sections were cut through the SCN on a freezing microtome and stored in 10 mM phosphate buffer in 0.9% saline. Adjacent sections were mounted on gelatin coated slides and stained with cresyl violet, or processed for immunohistochemical identification of arginine vasopressin-like immunoreactivity (VP-LI) (Incstar Science Stillwater, MN) utilizing the Avidin/Biotin system (Vector Laboratories, Burlingame, CA) (12).

Power spectral analysis employing a fast fourier transform (FFT) was applied to the data from each output for 10 days before surgery, 10 days after the surgery in LD 12:12 and 10 days in LL using 90-min average data intervals. The circadian peak was determined as that peak in the FFT corresponding most closely to 1 cycle/day. Because the amplitude of each output can affect the absolute power (7), peak height was not analyzed as a valid comparison between groups. Instead, the areas under the circadian portion (from 0.8 to 1.2 cycles/day) of the power spectral curves and under the entire plot were measured with a computer image analysis system (JAVA, Jandel Scientific, Corte Madera, CA). The area under the circadian peak was then divided by the total area and multiplied by 100 to compute the percentage of circadian power to total power.

Effects of Peripheral Blockade of Norepinephrine on Multiple Circadian Outputs

Adult male hooded Long-Evans rats (n=12) were maintained as described above. The cage tops were fitted with removable Plexiglas inserts, each of which contained a

stainless steel running wheel (27 cm in diameter by 12 cm wide). The rats were anesthetized as before and transmitters were implanted intraperitoneally. Therefore, wheel-running revolutions, as well as general activity, body temperature and heart rate were recorded simultaneously. Intraperitoneal (IP) implantation was used to prevent irritation of skin observed in some animals from the first experiment. Data for wheel running revolutions was collected from magnetic microswitches attached to the running wheels. Data from all four outputs was collected using Dataquest III as before.

After implantation of the transmitters, the rats were allowed to entrain to a 12:12 LD cycle for 14 days before being placed into the same dim LL as previously describe. After allowing them to free-run in LL for 10 days, subcutaneous injections of guanethidine (20 mg/kg) were given to 7 animals and saline was given to 5 animals once a day at randomized times for 8 days. The rats were then allowed to free run for another 20 days before being sacrificed.

Power spectral analysis was used exactly as before except in this case data was taken for 8 days before, during and after injections of guanethidine or saline. Differences in circadian power were determined using Mann-Whitney Wilcoxon *U* test.

Effects of Guanethidine on Central and Peripheral Norepinephrine Content

Rats (N = 12 males) were maintained in a separate room and simultaneously received the same injection treatments (6 received saline and 6 received guanethidine) as rats in Experiment 2. However, they were not implanted with transmitters and did not have access to running wheels. After the injections were ceased, the rats were anesthetized as

before and the following tissues were removed from each animal and placed on dry ice for quick freezing: 1) Peripheral tissues - spleen, lung, kidney, liver, adrenal, right atrium, pineal; 2) Brain areas - midbrain, striatum, hypothalamus, thalamus, cortex and cerebellum. After freezing, each tissue sample was placed in a preweighed 1.5 ml polypropylene microfuge tube and stored -90° C until processing. One week later samples were weighed to within 0.001 mg and all brain and peripheral tissues were sonicated in 400 μl of chilled dihydroxybenzylamine (DHBA, 62.5 ng/ml - internal standard) in perchloric acid (PCA) (exceptions: pineal - 100 μl of 62.5 ng/ml DHBA and adrenal 1 ml of 3000 ng/ml DHBA in PCA). These samples were alumina extracted and assayed for norepinephrine, epinephrine and dopamine using high-performance liquid chromatography with electrochemical detection (HPLC-EC) (13,32). Statistical significance between guanethidine and saline treated animals was determined using Student's *t* test.

Results:

Effects of SCN Ablation on Multiple Circadian Outputs

General activity, body temperature, and heart rate rhythms were all abolished by complete SCN lesions in both LD and LL (Figures 5, 6; Table 1). One rat showed disrupted rhythmicity in all three rhythmic outputs initially after the lesion but recovered circadian patterns later (Figures 3, 4). All SHAM rats showed robust rhythms in all measures (Figures 1, 2; Table 1).

Histological examination revealed VP containing cells in the dorsal-medial aspect of the SCN in all of the SHAM operated rats (54). Adjacent sections stained with cresyl violet also revealed intact SCN in all of these animals. Complete lesions, verified by both histological techniques, abolished circadian rhythmicity in all three outputs. A few VP immunoreactive cells were observed in the area of the SCN indicative of an incomplete lesion in the rat that showed a return of rhythmicity.

Effects of Peripheral Blockade of Norepinephrine on Multiple Circadian Outputs

In this experiment general activity, body temperature and wheel running rhythms were all unaffected by daily injections of guanethidine (Figures 9, 10; Table 2). However, guanethidine caused a significant reduction ($P < 0.05$) in the percent power of the circadian rhythm of heart rate (Figures 9, 10; Table 2). Rhythmicity was not completely restored after the daily injections were ceased, which is consistent with the known mechanism of action of guanethidine (66). No effect of saline injection was observed in any of the four rhythmic outputs either before, during or after injections (Figures 7, 8; Table 2).

Effects of Guanethidine on Central and Peripheral Norepinephrine Content

HPLC-EC analysis of central and peripheral tissues revealed a significant reduction ($P < 0.01$) in NE content in most peripheral tissues obtained from GUAN-injected rats (Table 3). NE levels were significantly increased in adrenal (Table 3). This result was expected, since responses that involve the release of amines from this site may be unaffected or even augmented by GUAN (66). No effect of the drug was seen in any central structure with the exception of hypothalamus, in which levels were elevated (Table 3).

Discussion:

Although behavioral, endocrinological and physiological rhythms (14, 35, 56, 63) have been shown to be under the control of the SCN, very little is known about the specific anatomical nature of the pathways that actually carry circadian signals to specific output systems. More is known about the anatomical connection between the pacemaker in the SCN and its control of the rhythm of pineal melatonin release than any other measurable rhythm regulated by the SCN. SCN neurons are known to project to the PVN (5, 58, 60, 65,), and this is a vital pathway in the rhythmic production of melatonin (22, 28, 53, 68). Cells from the PVN project through the medial forebrain bundle to the intermediolateral cell column of the spinal cord (53, 59), and these projections innervate preganglionic cells that innervate the cervical sympathetic chain including the superior cervical ganglion (SCG) (44, 45), which in turn sends noradrenergic post-ganglionic fibres to a variety of cephalic structures including the pineal gland (3, 8). Sympathetic terminals of the SCG release norepinephrine (NE) in a rhythmic fashion such that NE turnover is greater at night than during the day (9). High nocturnal NE release stimulates β-adrenoceptors post-synaptically to induce the synthesis of melatonin (10, 15).

In this study, we have shown that the circadian rhythms of general activity, body temperature and heart rate rhythms were all abolished by complete lesion of the SCN in both LD and in dim LL (Figures 5 & 6; Table 1). SHAM rats retained robust circadian rhythmicity in all measured outputs (Figures 1 & 2; Table 1), and a partially lesioned rat was initially disrupted but eventually became rhythmic in all three parameters (Figures 3 & 4 and Table 1). We have found that GUAN, which radically depletes norepinephrine stores

in peripheral tissues but not in brain regions (Table 3), selectively decreased the percent circadian power of the circadian rhythm of heart rate, whereas wheel running, general activity and body temperature were not affected (Figures 9 & 10 and Table 2). Moreover, the fact that GUAN selectively reduced percent circadian power in heart rate and significantly decreased mean heart rate (SAL - 378 beats/minute versus GUAN - 321 beats/minute· $^{D}<0.01$) suggests that the projection from the SCN to the sympathetic chain constitutes a ajor part of the pathway by which circadian information reaches peripheral sites.

The lesion study reported here verifies a previous study (49) which found that SCN lesions abolished the circadian rhythm of heart rate. The authors of this study suggested, although not specifically stated, that following lesions the mean level of heart rate was decreased to baseline levels which indicated that the sympathetic component of the rhythm had been removed. Although in the present study, there is a trend toward lower mean heart rate (385 beats/minute - SHAM versus 365 beats/minute - SCNX), our rats did not show a significant decrease in mean heart rate after complete lesion.

We cannot, at this point, rule out the possibility of circadian information being relayed via parasympathetic innervation, as well as sympathetic regulation. In fact, several authors have found, using the retrogradely transported fluorescent dye (bisbenzimide-true blue), that cells in the paraventricular nucleus of the hypothalamus (PVN) project to autonomic centers in the brainstem (specifically the dorsal vagal complex) and spinal cord of rats (52, 59). Further, the SCN is known to have dense projections into the sub-paraventricular zone and some fibers into the PVN (5, 58, 60, 65). This leaves open the

possibility that some of the circadian signal reaches the heart via parasympathetic input originating at the level of the SCN, and this is an area of current research interest.

The fact that the circadian rhythm of heart rate is selectively affected in this experiment is of particular interest. One might argue that under normal physiological conditions, the circadian rhythm of heart rate is being driven by the rat's activity rhythm. In tissue culture, rat heart cells have been reported to retain their circadian rhythmicity (61), and human heart transplant experiments indicate that both recipient and donor hearts show circadian periodicity (30) suggesting the possibility of a separate circadian oscillator within the heart. Our observation suggests that SCN lesion is removing the circadian signal to the heart specifically, indicating the presence of a distinct circadian pathway from the SCN to the heart.

This circadian regulation may be of some biomedical importance. There is a circadian pattern in the risk of several cardiovascular problems, such as myocardial infarction, sudden death, and stroke in humans (34, 37, 46, 62, 67). Some authors have suggested that the sympathetic nervous system plays a vital role in these events (37, 40). Heart rate (29), mean arterial pressure (34), and norepinephrine plasma levels (55) all rise sharply from minimum to maximum levels between six and nine every morning, and this may contribute to some pathologies. These data corroborate the view that circadian information from the SCN is reaching peripheral sites via sympathetic innervation, and that a better understanding of this pathway may lead to the prevention of a substantial number of cases of myocardial infarction and sudden cardiac death.

The effects of SCN lesions on the circadian rhythm of body temperature are

controversial, and the present study will not resolve the issue due to the relatively small numbers of animals. The method of SCN ablation, the sex, species, and strain within a species of the animals used, frequency of recording, postlesion conditions, and verification of surgeries have varied among reports making interpretation of results difficult. Some authors have reported that the body temperature rhythm remains, often with an altered appearance, after destruction of the SCN (17, 20, 21, 38, 43, 51). However, others have reported that SCN lesions result in the elimination of body temperature rhythmicity (1, 18, 19, 50, 57). The two most recent studies (26, 51) do not resolve the controversy as these results are variable. Satinoff and Prosser (1988) used male Long-Evans rats, automated continuous data collection of body temperature, drinking and activity, and Nissl stain to verify completeness of lesions. They report that complete lesions abolish drinking and activity rhythms but not body temperature rhythmicity. Kittrell (1991) reports that in rats that have no visible remaining SCN tissue as verified by both cresyl violet and cytochrome oxidase staining procedures, all measured rhythmicity (drinking, activity, and body temperature) was abolished. Our results support the idea that complete lesions must be verified by additional staining procedures. Complete lesions in the present study, as verified by both Nissl stain and VP-LI, abolish rhythmicity in all three measures. Further, in the single animal receiving a partial lesion, circadian rhythmicity was retained with less percent circadian power.

It is interesting that GUAN has no effect on the circadian rhythm or absolute value of body temperature. This suggests that, under these laboratory conditions, sympathetic activity contributes little to the generation of body temperature rhythms.

In conclusion, our results confirm the idea that the SCN is the master pacemaker controlling the circadian rhythms of activity, body temperature and heart rate. We have also demonstrated that the circadian rhythm of heart rate can be selectively affected by a peripheral sympathetic blockade induced by guanethidine. These data suggest that the pathway by which information travels from the SCN to the heart shares with the pathway to the pineal gland the sympathetic chain. Our results raise the possibility that other peripheral organs may receive circadian information originating in the SCN via the sympathetic nervous system.

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Table 1 - Percent Circadian Power (PCP) of SHAM and SCN lesioned Rats

TREATMENT - RHYTHM	PCP ± SD (LD 12:12) BEFORE SURGERY	PCP ± SD (LD 12:12) AFTER SURGERY	PCP ± SD (DIM LL) AFTER SURGERY
SHAM - BODY TEMP.	43.92 ± 7.76	40.37 ± 5.81	36.44 ± 10.28
SHAM - GENERAL ACTIVITY	29.92 ± 7.21	27.83 ± 9.96	26.94 ± 10.65
SHAM - HEART RATE	23.00 ± 8.06	23.61 ± 9.36	23.22 ± 10.33
SCNX - BODY TEMP	42.66 ± 5.93	11.19 ± 3.68 *	11.15 ± 3.04 *
SCNX - GENERAL ACTIVITY	33.59 ± 3.74	7.70 ± 4.82 *	9.95 ± 6.07 *
SCNX - HEART RATE	29.30 ± 6.31	9.00 ± 3.67 *	8.90 ± 5.80 *

* $P < 0.05$ versus percent circadian power (LD 12:12) before surgery

Table 2 - Percent Circadian Power (PCP) of Guanethidine and Saline Injected Rats

TREATMENT - RHYTHM	PCP ± SD (DIM LL) BEFORE INJECTIONS	PCP ± SD (DIM LL) DURING INJECTIONS	PCP ± SD (DIM LL) AFTER INJECTIONS
GUAN - BODY TEMP	45.96 ± 7.99	40.56 ± 7.66	33.72 ± 17.10
GUAN - WHEEL RUNNING	36.37 ± 3.50	34.25 ± 3.19	33.14 ± 6.49
GUAN - GENERAL ACTIVITY	35.40 ± 7.68	32.06 ± 4.36	30.46 ± 12.31
GUAN - HEART RATE	24.99 ± 12.24	8.14 ± 5.94 *	11.59 ± 7.73
SAL - BODY TEMP	45.31 ± 6.61	44.08 ± 6.29	36.38 ± 13.01
SAL - WHEEL RUNNING	27.63 ± 9.43	26.84 ± 7.49	28.64 ± 4.56
SAL - GENERAL ACTIVITY	29.47 ± 9.51	31.60 ± 4.00	32.76 ± 4.61
SAL - HEART RATE	20.77 ± 5.13	20.25 ± 7.03	19.21 ± 6.80

* $P < 0.05$ versus percent circadian power (DIM LL) before injections

Table 3 - Effects of Guanethidine on Central and Peripheral Norepinephrine Content

STRUCTURE	NE CONTENT (NG/G ± SE) SALINE TREATED	NE CONTENT (NG/G ± SE) GUANETHIDINE TREATED
MIDBRAIN	630.0 ± 52.0	621.0 ± 64.0
STRIATUM	365.5 ± 32.0	366.0 ± 73.0
HYPOTHALAMUS	1361.0 ± 82.0	1759.0 ± 135.0 *
THALAMUS	419.6 ± 37.0	535.0 ± 46.0
CORTEX	275.7 ± 15.0	281.9 ± 16.0
CEREBELLUM	264.5 ± 17.0	250.9 ± 30.0
SPLEEN	2699.0 ± 316.0	31.0 ± 7.5 *
LUNG	138.2 ± 21.0	< 0.017 *
KIDNEY	316.4 ± 11.0	43.7 ± 15.0 *
LIVER	68.1 ± 5.6	10.4 ± 1.2 *
ADRENAL	45990.0 ± 5731.0	80188.0 ± 8040.0 *
RIGHT ATRIUM	1988.4 ± 36.0	22.7 ± 6.4 *
PINEAL	1.957 ± 0.32 NG/GLAND	0.389 ± 0.092 NG/GLAND *

* $P < 0.01$ versus saline treated

Figure Legends

Figure 1: Actograms from a SHAM operated rat showing robust rhythmicity in three rhythmic outputs: A) body temperature, B) general activity, and C) heart rate. * denotes the day of surgery. The days immediately following the * are in the same light:dark cycle (LD 12:12) as before the surgery, and all three outputs free run in dim LL.

Figure 2: Power spectral analyses are shown for data from each output for 10 days before a SHAM surgery, and 10 days in dim LL following the surgery. Body temperature, general activity, and heart rate rhythms all have large peaks in the circadian range both before and after surgery.

Figure 3: Actograms of the rat with a partial lesion causes an initial disruption in circadian rhythmicity of: A) body temperature, B) general activity, and C) heart rate. * denotes the day of surgery. Some disruption of rhythmicity is seen in all three outputs immediately following the surgery, but rhythmicity returns both in LD 12:12 and dim LL.

Figure 4: Power spectral analyses are shown for data from each output for 10 days before a partial SCN lesion, and 10 days in dim LL following the surgery. Body temperature, general activity, and heart rate rhythms all retain large peaks in the circadian range before and after surgery.

Figure 5: Actograms of a rat receiving a complete SCN lesion shows disrupted rhythmicity

in three rhythmic outputs: A) body temperature, B) general activity, and C) heart rate. * denotes the day of surgery. The days immediately following the * are in the same light:dark cycle (LD 12:12) as before the surgery, and all three outputs are disrupted both in the LD 12:12 and in dim LL.

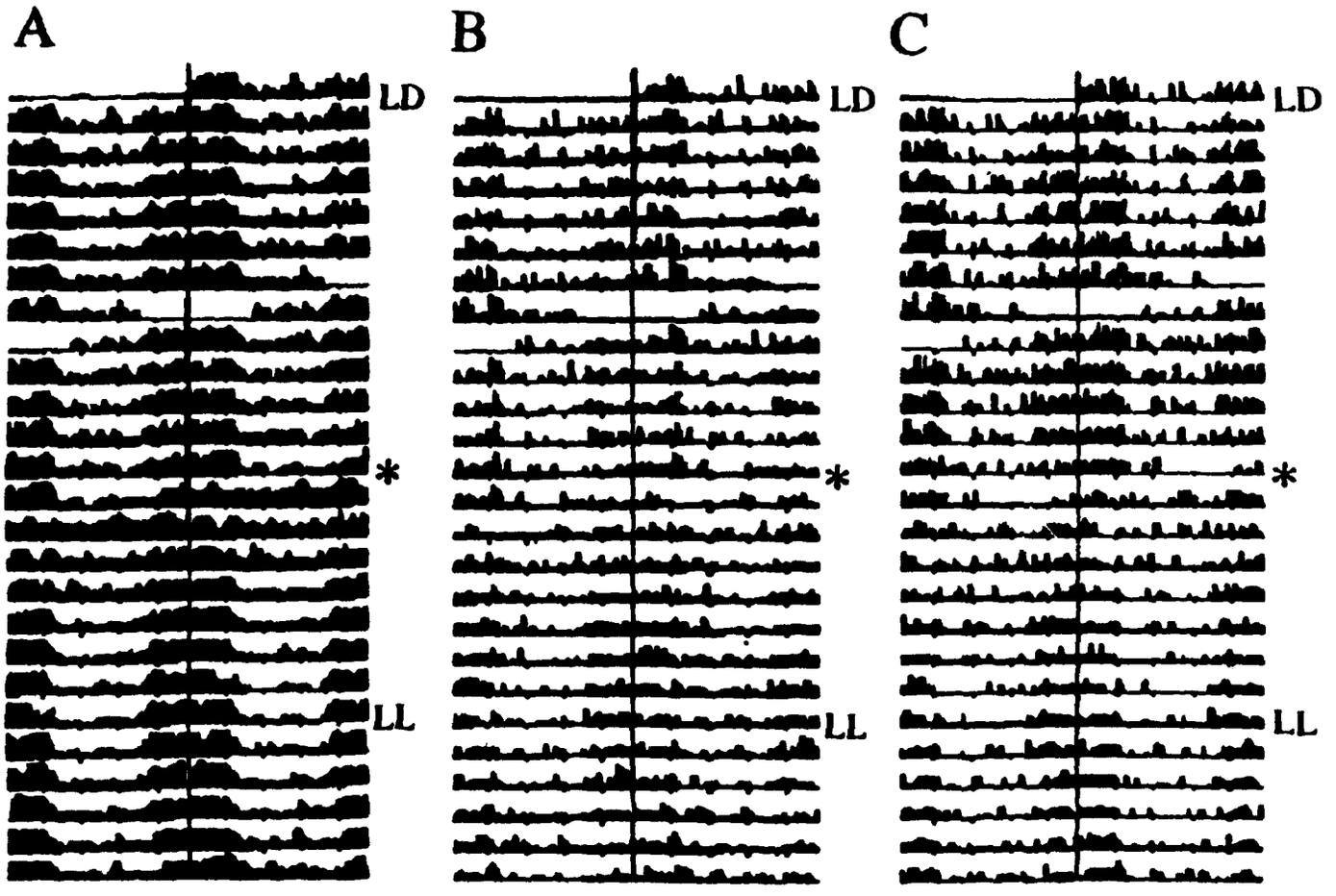
Figure 6: Power spectral analyses are shown for data from each output for 10 days before a complete SCN lesion, and 10 days in dim LL following the surgery. Circadian power of body temperature, general activity, and heart rate was reduced by surgery.

Figure 7: Analogue plots of four days of data from a rat before receiving saline injections (A, C, E, G) and during the saline injections (B, D, F, H). A & B represent body temperature, C & D wheel running, E & F general activity, and G & H represent heart rate data.

Figure 8: Power spectral analyses from the data indicated in Figure 7. Circadian power was maintained throughout.

Figure 9: Analogue plots of four days of data from a rat before receiving guanethidine injections (A, C, E, G) and during the guanethidine injections (B, D, F, H). A & B represent body temperature, C & D wheel running, E & F general activity, and G & H represent heart rate data.

Figure 10: Power spectral analyses from the data indicated in Figure 9. Circadian power was reduced in heart rate only by guanethidine.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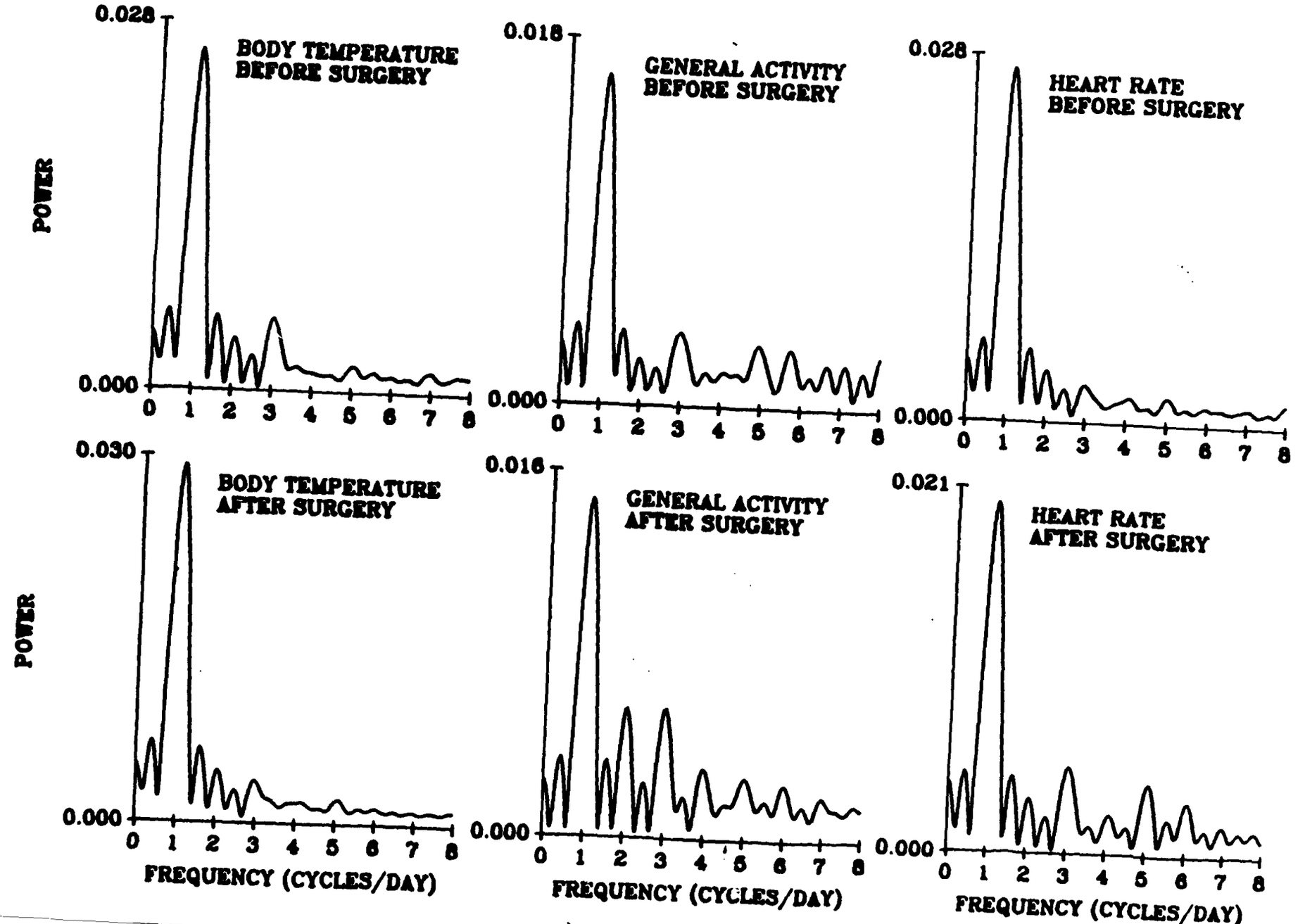
B
C
LD
LD
LD
*
*
*
LL
LL
LL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GENERAL ACTIVITY
BEFORE SURGERY
HEART RATE
BEFORE SURGERY
BODY TEMPERATURE
AFTER SURGERY
GENERAL ACTIVITY
AFTER SURGERY
HEART RATE
AFTER SURGERY
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POWER
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0.000
0.018
0.000
0.028
0.000
0.030
0.000
0.016
0.000
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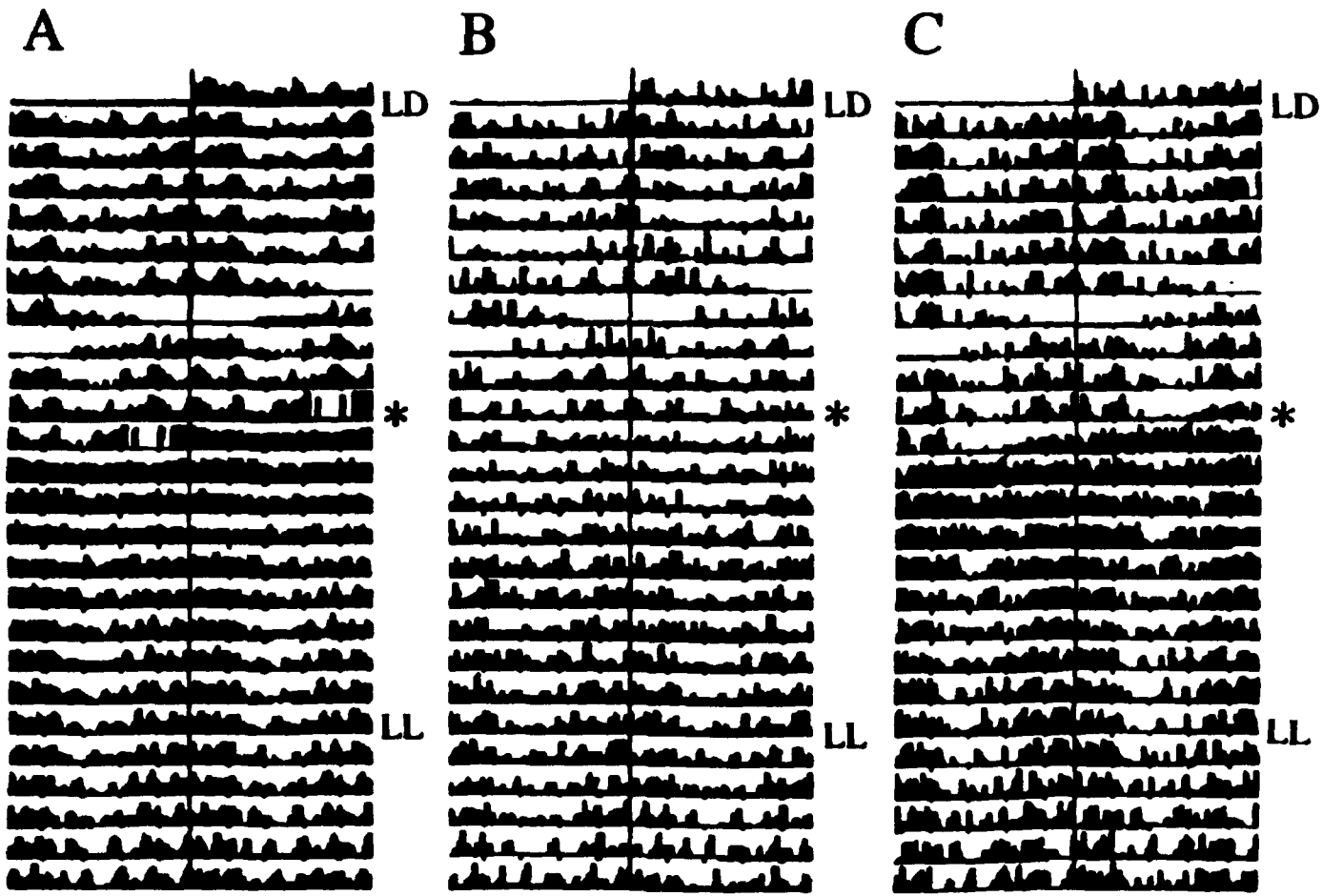
FREQUENCY (CYCLES/DAY)
FREQUENCY (CYCLES/DAY)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C
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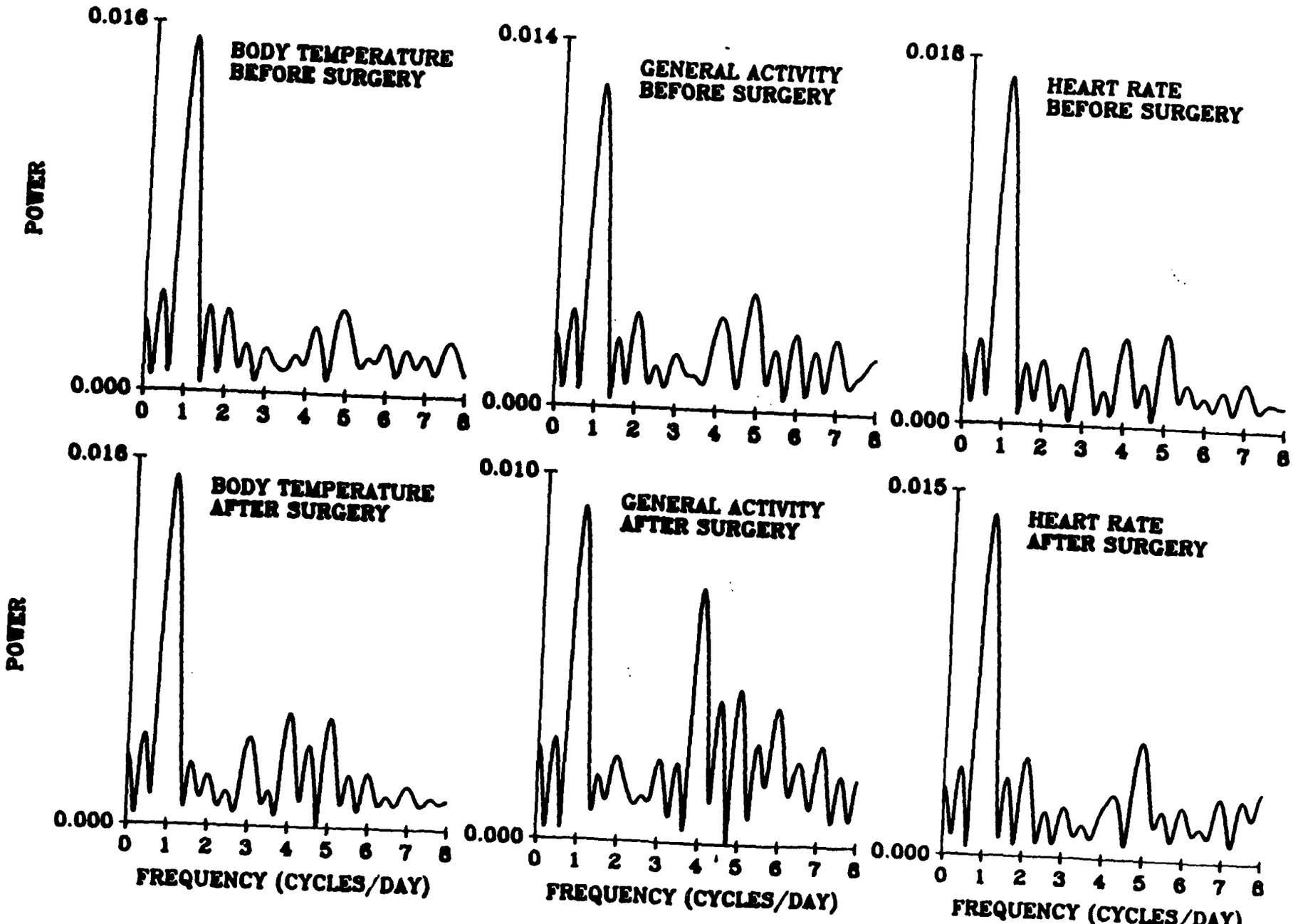
BODY TEMPERATURE BEFORE SURGERY
GENERAL ACTIVITY BEFORE SURGERY
HEART RATE BEFORE SURGERY
BODY TEMPERATURE AFTER SURGERY
GENERAL ACTIVITY AFTER SURGERY
HEART RATE AFTER SURGERY
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POWER
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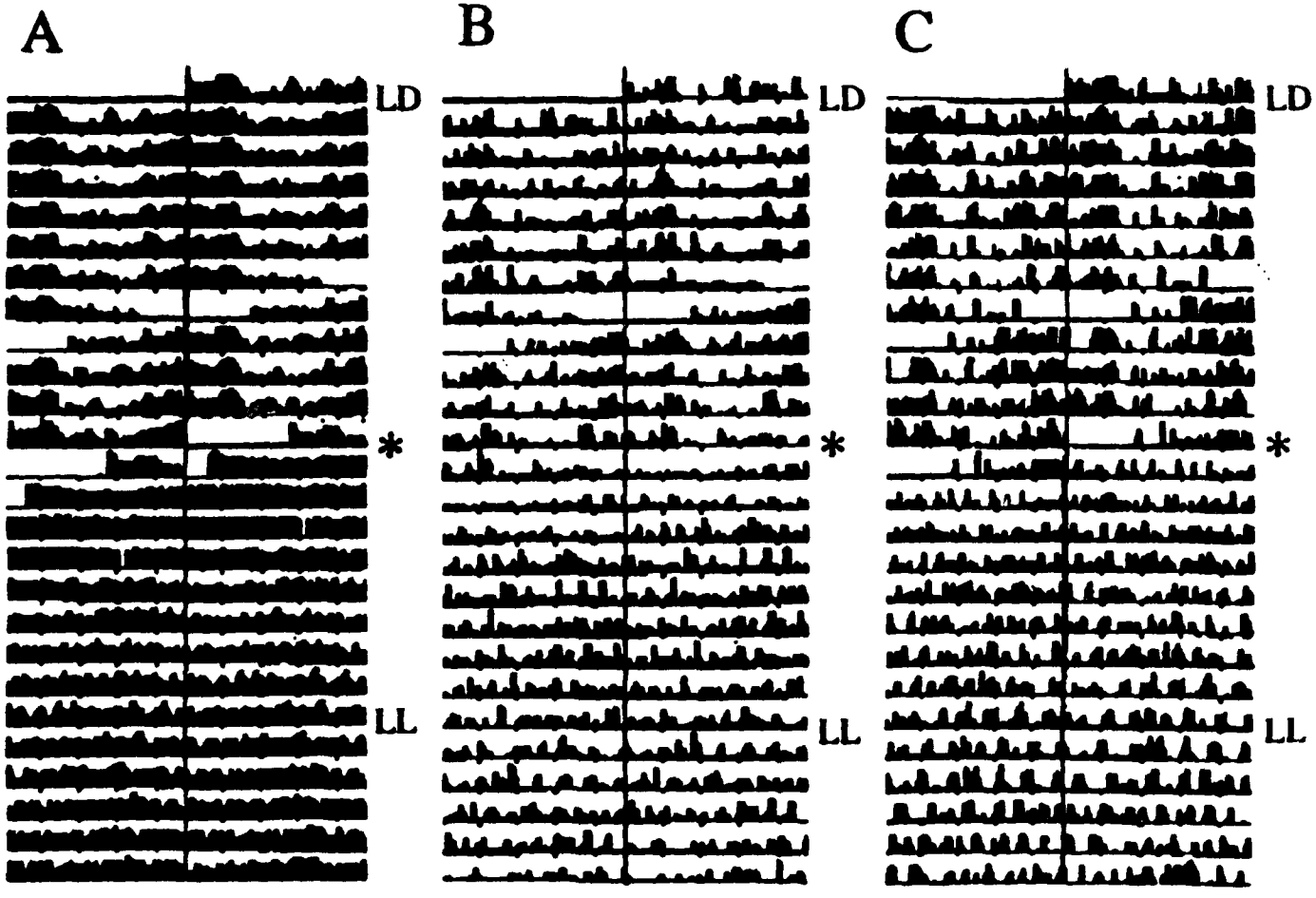
FREQUENCY (CYCLES/DAY)
FREQUENCY (CYCLES/DAY)
FREQUENCY (CYCLES/DAY)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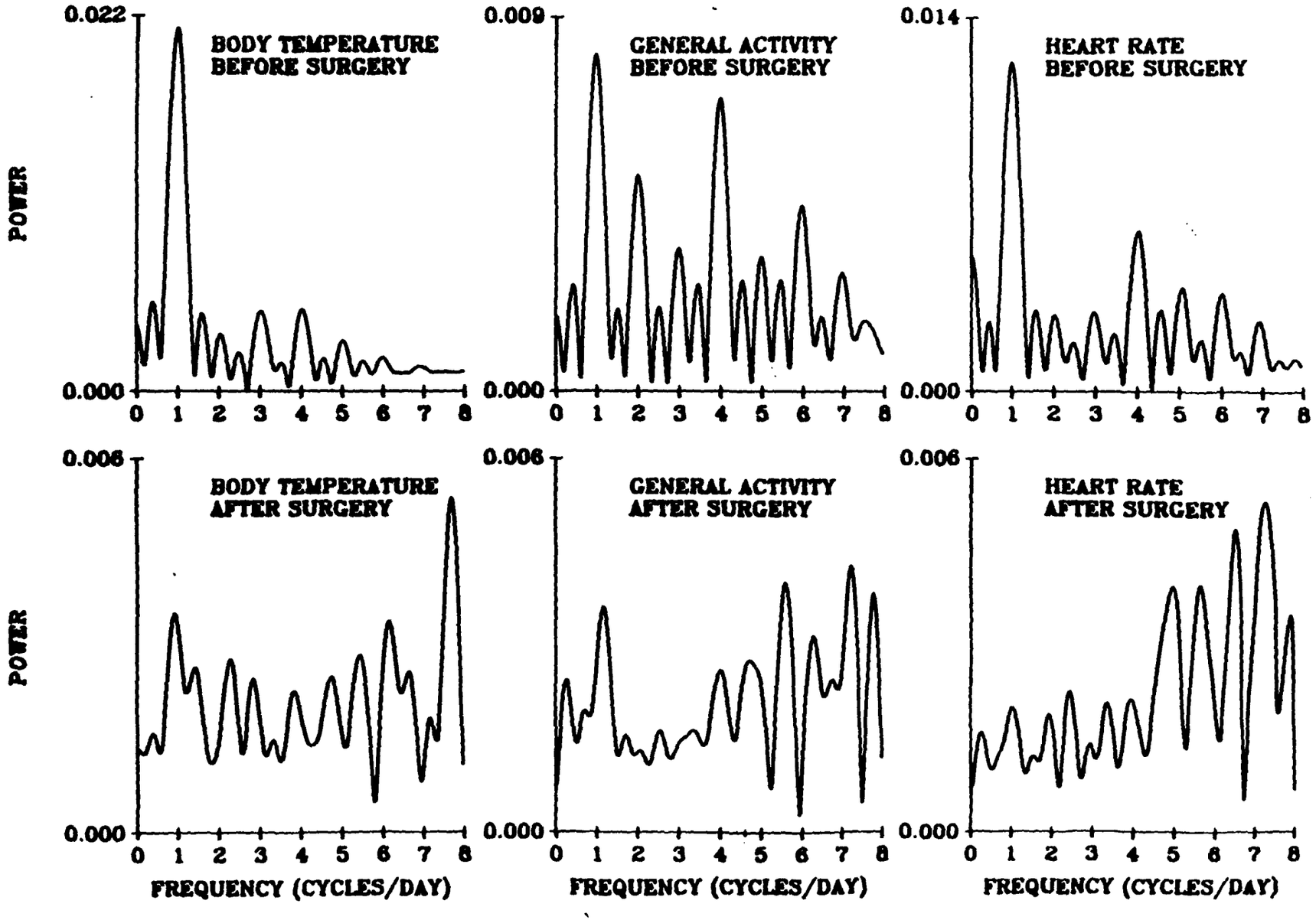
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GENERAL ACTIVITY
BEFORE SURGERY
HEART RATE
BEFORE SURGERY
BODY TEMPERATURE
AFTER SURGERY
GENERAL ACTIVITY
AFTER SURGERY
HEART RATE
AFTER SURGERY
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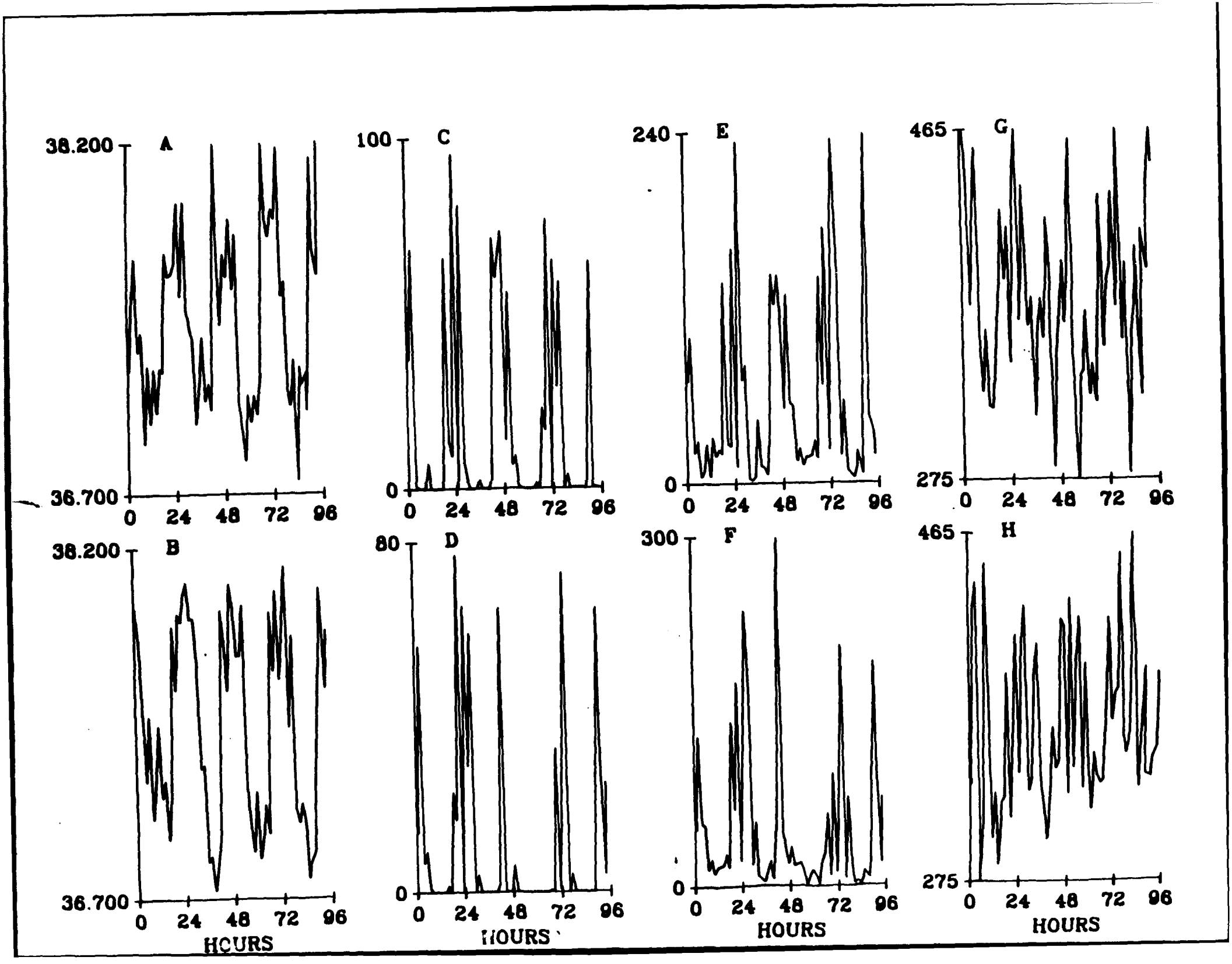
FREQUENCY (CYCLES/DAY)
FREQUENCY (CYCLES/DAY)
FREQUENCY (CYCLES/DAY)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C
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G
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38.200
B
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HOURS
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HOURS
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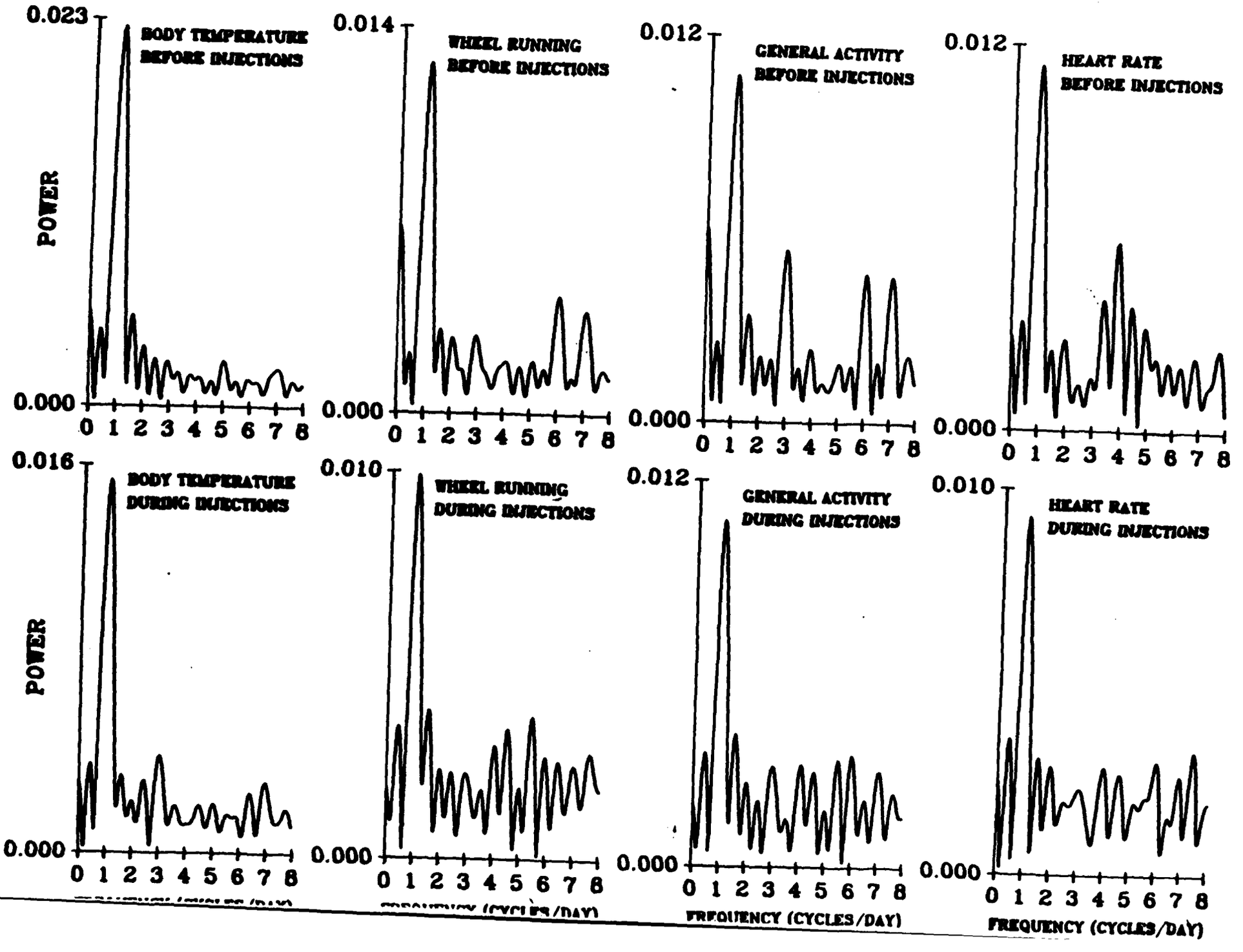
HOURS
465
H
275
0 24 48 72 96
HOURS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WHEEL RUNNING
BEFORE INJECTIONS
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GENERAL ACTIVITY
BEFORE INJECTIONS
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0.012
HEART RATE
BEFORE INJECTIONS
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0 1 2 3 4 5 6 7 8
0.016
BODY TEMPERATURE
DURING INJECTIONS
POWER
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WHEEL RUNNING
DURING INJECTIONS
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GENERAL ACTIVITY
DURING INJECTIONS
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0 1 2 3 4 5 6 7 8
FREQUENCY (CYCLES/DAY)
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HEART RATE
DURING INJECTIONS
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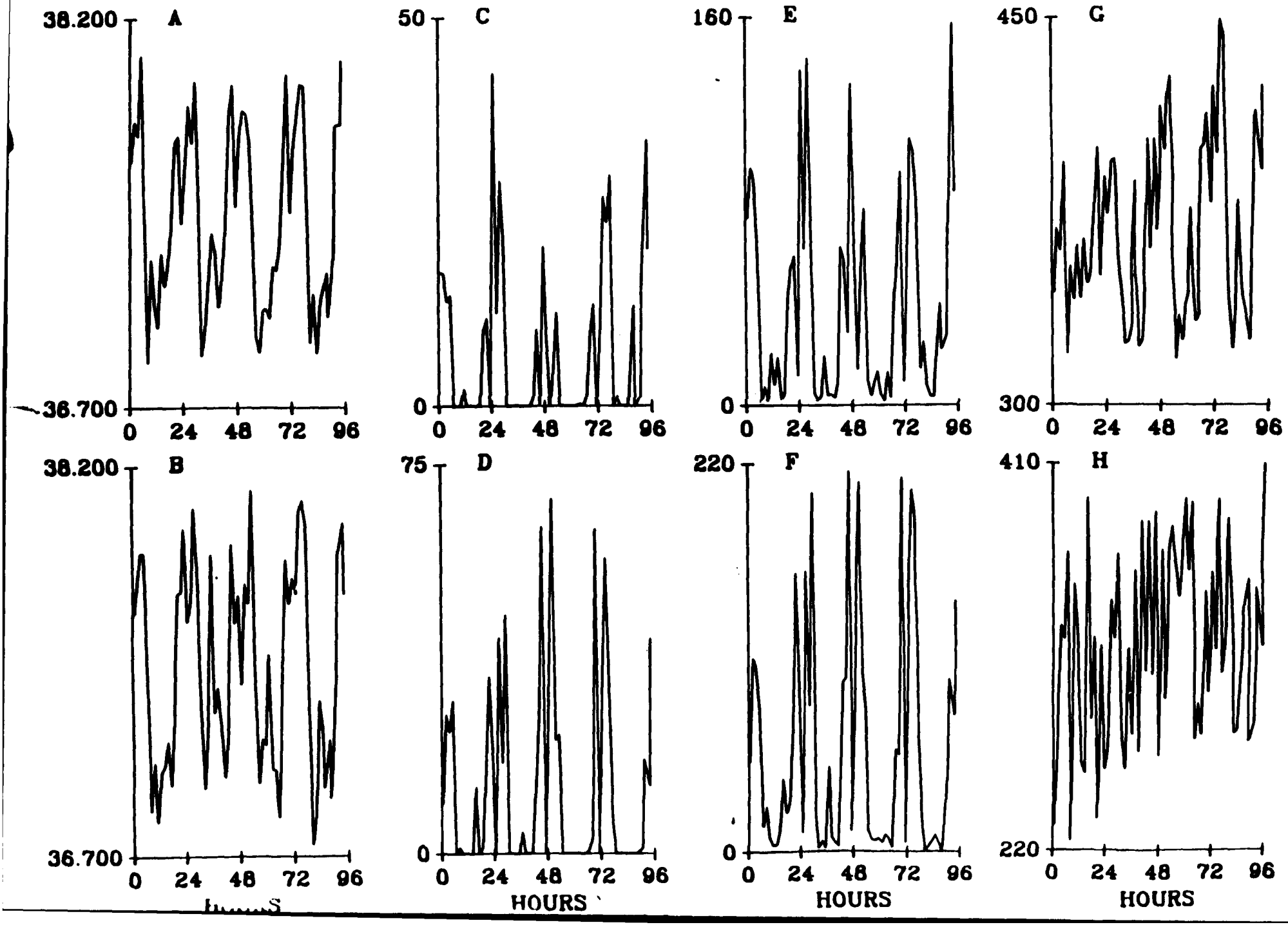
FREQUENCY (CYCLES/DAY)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C
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300
0 24 48 72 96
38.200
B
36.700
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HOURS
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HOURS
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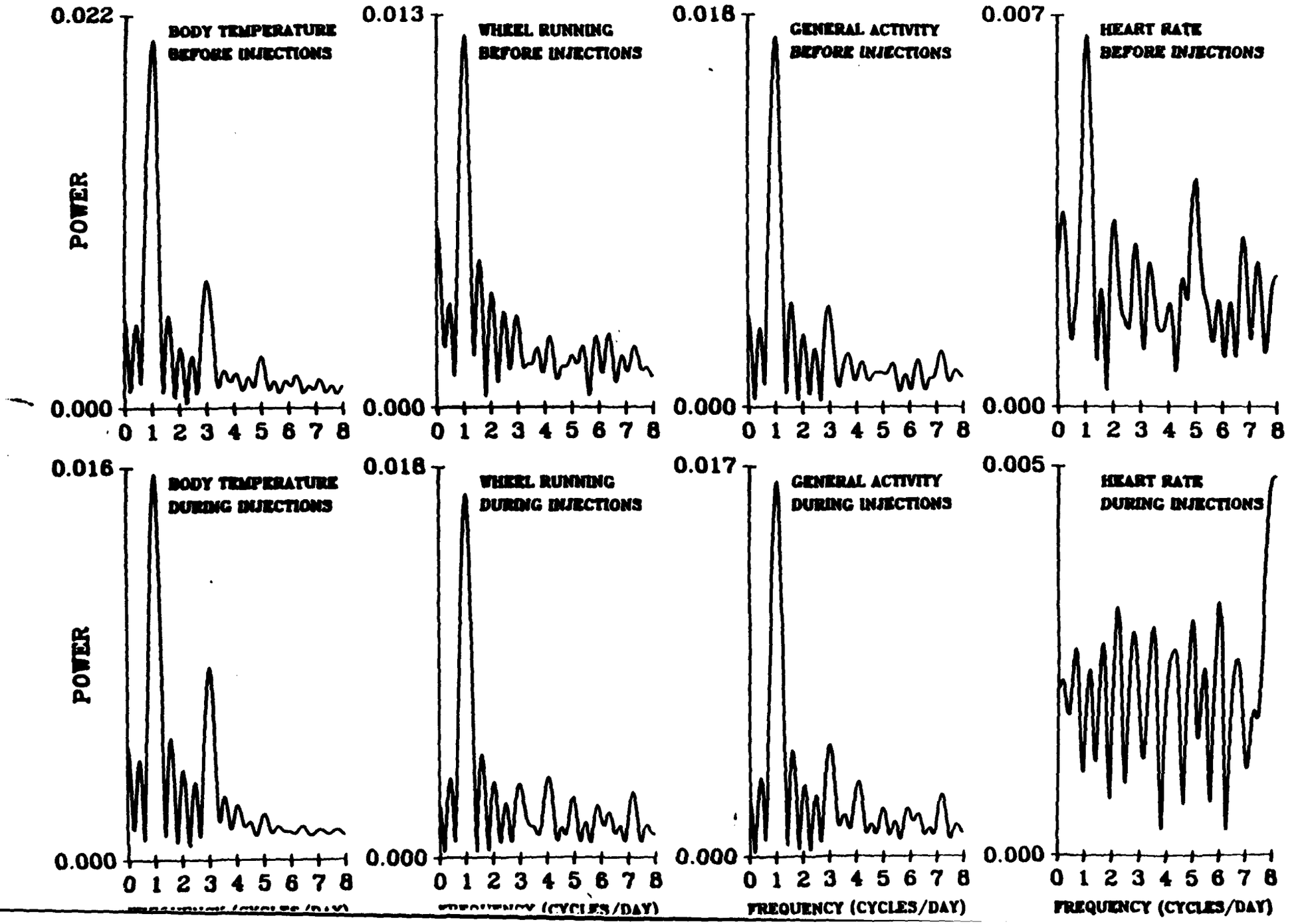
220
0 24 48 72 96
HOURS


BODY TEMPERATURE
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WHEEL RUNNING
BEFORE INJECTIONS
GENERAL ACTIVITY
BEFORE INJECTIONS
HEART RATE
BEFORE INJECTIONS
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DURING INJECTIONS
WHEEL RUNNING
DURING INJECTIONS
GENERAL ACTIVITY
DURING INJECTIONS
HEART RATE
DURING INJECTIONS
POWER
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